STUDIES ON NEUROPROTECTIVE ACTION OF A BIOFLAVANOID IN LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION

Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

CHENNAI- 600 032

In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch-IV -- PHARMACOLOGY

Submitted by

Name: ANOOP. A. P

REG.No.261425217

Under the Guidance of

Dr. R. SHANMUGA SUNDARAM, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACOLOGY

J.K.K. NATTARAJA COLLEGE OF PHARMACY

KUMARAPALAYAM – 638183

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CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"STUDIES ON NEUROPROTECTIVE ACTION OF A BIOFLAVANOID IN LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION",** submitted by the student bearing **Reg. No: 261425217** to "**The Tamil Nadu Dr. M.G.R. Medical University – Chennai**", in partial fulfilment for the award of Degree of **Master of Pharmacy** in **pharmacology** was evaluated by us during the examination held on……

Internal Examiner **External Examiner**

CERTIFICATE

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Dr. R. Shanmuga Sundaram, M.Pharm.,
GUIDE

Dr. R. Sambathkumar, M. Pharm., PhD.,
Principal

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DECLARATON

I do hereby declared that the dissertation **"STUDIES ON NEUROPROTECTIVE ACTION OF A BIOFLAVANOID IN LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION"** submitted to "**The Tamil Nadu Dr. M.G.R Medical University - Chennai",** for the partial fulfilment of the degree of **Master of Pharmacy** in **pharmacology,** is a bonafide research work has been carried out by me during the academic year 2016-2017, under the guidance and supervision of **Dr. R. Shanmuga Sundaram, M.Pharm., PhD.,** Professor & Head, Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Kumarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

Place: Kumarapalayam **Mr. ANOOP. A. P**

Date: **Reg.no. 261425217**

Dedicated to Parents, Teachers & My Family

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Reg.no. 261425217

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1. Introduction

I.a. Stress

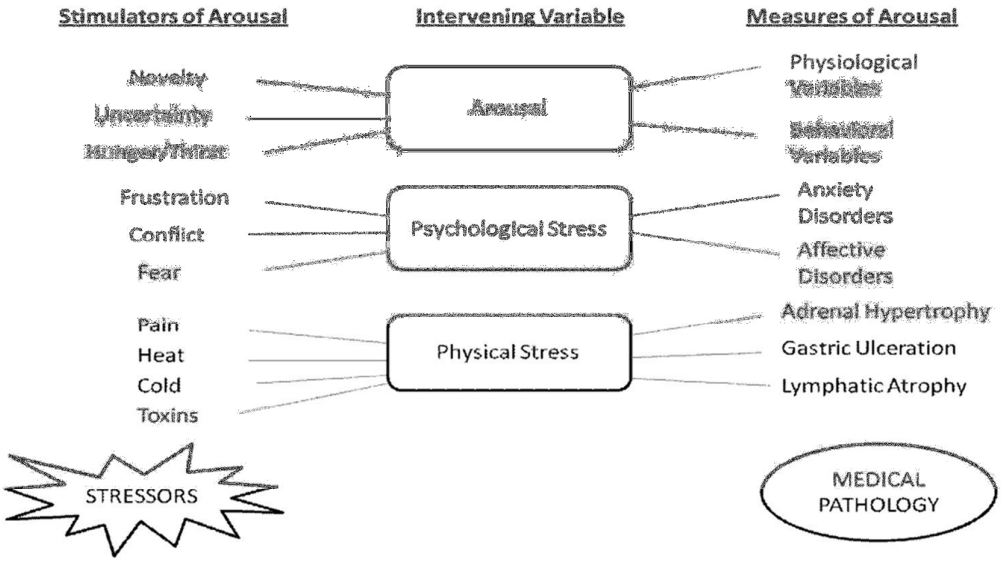
Stress is a common experience of daily life and all organisms have developed mechanisms to cope with it. Sustained stress can have numerous pathophysiological effects such as activation of neuro–endocrine [limbic–hypothalamic–pituitary adrenal system] (*Bonfiglio et al., 2011*) and hormonal (corticosterone release) functions (*Fuchs & Flugge, 1998*). Sustained and persistent stressful conditions can precipitate anxiety and affective disorders such as depression, which further leads to the excessive production of free radicals and oxidative burden (*Maes et al., 2011*).

Stressful events can activate the Hypothalamo-Pituitary-Adrenal (HPA) axis (*Kvetnansky et al., 2002*) and increase the release of Corticotrophin Releasing Hormone (CRH) from the hypothalamic paraventricular nucleus, causing the secretion of adrenocorticotropin (ACTH) from anterior pituitary, which in turn stimulates the secretion of glucocorticoids from the adrenal cortex (*Pacak et al., 1993; Venihaki et al., 1997*). Glucocorticoids possess broad spectrum of actions affecting expression and regulation of genes throughout the body readying the organism for changes in energy and metabolism required for coping (*Akil & Morano, 1995; Levine, 2005*). Stress has been postulated to be involved in the etiopathogenesis of a variety of disease state including hypertension, coronary heart disease (*Roy et al., 2001*), gastric ulcers (*Yadin & Thomas, 1996*), diabetes (*Fitzpatrick et al., 1992*), immuno-suppression (*Purret, 2001*), mental depression, memory loss (*Gareri et al., 2000*), and host of other diseases. The resultant disturbances may vary depending upon type, intensity, and the duration of a particular stressor and the strain\sex differentiation of the subjects (*Kioukia-Fougia et al., 2002*). Different animal models for stress have been developed and used frequently to evaluate the anti-stress activity of compounds of both natural and synthetic origin.

In an organism, diverse stressors activate a wide spectrum of interacting hormonal and neuronal systems resulting in behavioral (anxiety disorders, decrease in food intake, decrease in sexual behavior, and loss of cognitive function) and physiological responses [activation of pituitary adrenal axis and release of glucocorticoids into the blood stream] (*Henry & Stephens, 1977*). These stressors are stimulators of arousal and lead to autonomic (changes in body temperature and tachycardia) and behavioral changes; however, when arousal increases to stress-like levels, it results in psychiatric and physical disorders (*Hennessy et al., 1979*)

[Figure 1].Different animal models have been developed for chronic stress induced neurological disorders such as the olfactory bulbectomy model, and the chronic unpredictable stress model. These animal models are used to screen various new chemical entities and to develop a better understanding of the underlying molecular pathway in chronic stress pathology. Stress responses are variable and there are individual differences both physiologically and behaviorally in how an organism perceives a perturbation and in the resulting adaptational/maladaptational processes (*Weiner, 1992*).

Figure 1: Relationship between arousal, psychological stress, physical stress and pathology



Stressor is a stimulus, either internal or external, that activates the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system resulting in physiological change (*Maier & Watkins, 1998*). Long-term exposure to stressors can cause depression, (*Nirmal et al., 2008*) post-traumatic stress disorder, and anxiety disorders. The degree of behavioral control that an individual has over a stressor often determines the consequence of that stressor and plays a key role in the development of pathological behaviors after a traumatic event (*Christianson et al., 2009*). The potency to cope with the stressors is a fundamental requirement for survival. Brain is the target for different stressors because of its high sensitivity to stress-induced degenerative conditions (*Sahin&Gumuşlu, 2007*). The brain tissue is made up of large amounts of polyunsaturated fatty acid, thus making it vulnerable to free radical attacks (*Gutteridge, 1995*).

I.b. Consequences of Stress

Normal development and preservation of life and species depend on a normally functioning stress system. Maladaptive neuroendocrine responses, i.e., dysregulation of the stress system, may lead to disturbances in growth and development, and cause psychiatric, endocrine/metabolic, and/or autoimmune diseases or vulnerability to such diseases.

I.c. Stress and anxiety

According to previous reports stress induces anxiety-like behavior in both humans and animals (*Liezmann et al., 2011*). In response to stress, there is an increase in CRF levels. The CRF level decreases when the stressor is no longer present. Lee, et al. reported that chronic stress increases the length and volume of expression of CRF in areas of the brain associated with fear and emotion, including the amygdale (*Lee et al., 2008*) [Figure 2]. Such chronic stress changes the body's response, and the resulting increased expression of CRF is believed to be the cause of health-related stress problems such as anxiety, depression, and infertility (*Kimura et al., 2010*). Exposure to stress represents an important factor for a number of neuropsychiatric disorders such as depression, post-traumatic stress disorder, and other anxiety disorders (*Horstmann& Binder, 2011*). There are earlier reports of enhanced noradrenergic or HPA axis activity in many psychopathological states such as depression and anxiety disorders (*Boyer, 2000; Kendler, 1996*).

Oxidative stress contributes toward neuronal degeneration in the central nervous system in the process of aging as well as neurodegenerative diseases (*Hovatta et al., 2010*). The production of reactive oxygen species (ROS) is greatly increased under many conditions of toxic stress (*Liu & Schubert, 2009*). One of the reasons for stress-induced enhancement of free radicals may be the elevation of nitric oxide (NO) production (*Matsumoto, 1999*). This is further supported by the present determination of nitrite levels, which revealed significant increase in brain NO levels in stressed mice. The reactive nitrogen species along with ROS, working in concert with an inflammatory process, may play a substantial role in the pathogenesis of depression (*Matsumoto, 1999*). Stress has been shown to be responsible for the depletion of several free radical detoxifying enzymes such as glutathione peroxidase, catalase, and superoxide dismutase (*Zaidi & Banu, 2004*).

This results in oxidative burden, which has been implicated in stress as well as in the pathogenesis of several disease states. Since brain tissues consist of a high content of PUFA and one of the important consequences of oxidative stress is peroxidation of membrane lipids, this reaction produces marked damage to the structure and function of cell membranes in

these tissues (*Jain et al., 1991*). Therefore, lipid peroxidation was supposed as the major biochemical alteration and consequence of oxidant-induced cell injury. Thus, the important consequences of stress could be attributed to stress-induced lipid peroxidation.

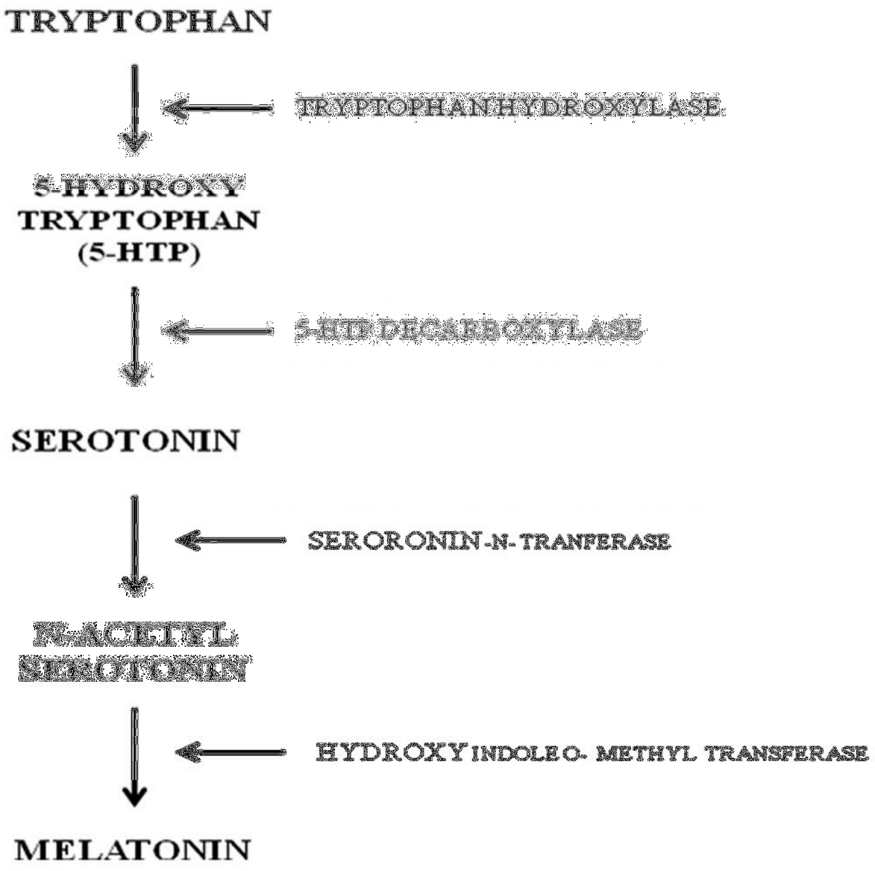


Figure 2: *De novo* synthesis pathway of melatonin.

I.d.Stress and immunological changes

Stress has been associated with impaired immune function and increased susceptibility to infectious diseases (*Connor & Leonard, 1998*). It is now believed that the nervous, endocrine, and immune systems are so intimately connected that they should be regarded as a single network rather than as three separate systems (*Connor & Leonard, 1998*). It is widely accepted that psychological stress and psychiatric illness can compromise immune function (*Leonard, 1995*) and soluble mediators released by immune cells can affect the central nervous system, thus producing alterations in behavior. Exposure to stressful life events such as academic examinations and divorce was reported to cause impairments in various aspects

of cellular immune function (*Bartrop et al., 1977*). There are also reports of immune activation, (*Bartrop et al., 1977*) in addition to immunosuppression in both the depressed and subjects exposed to stressful life events.

A requirement of all studies on stress is an adequate and appropriate animal model of stress. An ideal animal model should be able to reproduce each of the aspects of stress response and should be able to mimic the natural progression of the disease. However, none of the models available is able to entirely reproduce stress response. Some models reproduce physical stress and associated neuroendocrine changes (*Kvetnansky&Mikulai, 1970*), whereas others better reproduce the psychological stress and associated behavioral changes (*Marcelo et al., 2007*). Acute models do not reproduce the neuroendocrine dysfunction whereas a chronic model might be able to do so. Therefore, a correct model should be used to evaluate specific aspects of the stress response. Each model has inherent limitations including lack of stability, lack of predictability of tissue damage, and lack of adjustability. And hence a literature survey of more than 35 years (1970-2007) was conducted based on the description of the models, potential utilization of the models, and value of the models for testing of new medical interventions for the management of stress. The purpose of this review was to assess different models of stress.

I.e. Stress and Behavior

Stress can be defined generally as responses to demands upon the body (*Koob, 1999*). It is the body's reaction to a change that requires a physical, mental, or emotional adjustment or response (*Selye,1936*). It can come from any situation or thought that makes one feel frustrated, angry, nervous, or anxious. Conceptually, stress can be any threat,either real or perceived, to the well being of an organism and it can be of two types.Stress can be defined as "the generalized, non-specific response of the body to any factor that overwhelms, or threatens to overwhelm, the body's compensatory abilities to maintain homeostasis". The following stressors can induce a stress response: physical stressors (trauma, surgery, intense heat or cold); chemical stressors (reduced oxygen supply, acid-base imbalance); physiological stressors (heavy exercise, hemorrhagic shock, pain); psychological or emotional stressors (anxiety, fear, sorrow); and social stressors (personal conflicts, change in life style). Stressors can be short-term (acute stress) or occur on a daily basis (chronic stress). Reactions to Stressors have been suggested to be of several types, and those of most importance are the "active fight/flight" pattern (sympathetic adrenal modular system) and the "passive" pattern (pituitary-adrenal cortical system involving the hypothalamic-pituitary-

adrenal HPA axis) activation of the sympathetic adrenal medullar system, with release of catecholamine (adrenaline and nor adrenaline), is typical during periods of acute stress.

Hyper-activation of the HPA axis, with release of corticosteroids (cortisol), has been associated with individuals who are chronically stressed. Furthermore, it has been proposed that a hyperactive HPA axis may be programmed during the prenatal period as a result of foetal growth retardation. Responses to acute or chronic stress can lead to physiological changes which include slowed gastric emptying, elevation of blood pressure, increase in heart rate, mobilisation of energy stores, and decrease in blood flow to non-essential organs, for example the digestive system, kidneys and skin. Hormones released in response to stress can specifically affect appetite. Noradrenalin and corticotrophin-releasing hormone (CRH) have been reported to suppress appetite during stress, whereas cortical is known to stimulate appetite during recovery from stress. Anxiety, depression, uneasiness, anger, apathy and alienation are emotions that commonly accompany chronic stress. The responses to acute or chronic stress also include a number of modifying behaviours such alcohol consumption, smoking and eating. Immobilization, a severe stressor, consistently reduced ordinary food (rat chow) intake in rats when administered chronically and even acutely. This stress-Induced inhibition of feeding behaviour may have a physiological basis. As CRF levels increase in response to stress, food intake decreases. Furthermore, this wasconfirmed by a study in rats which found CRF inhibited the hyperphagia induced by neuropeptide Y (*Torres et al., 2007*).

Increased concentrations of HPA axis hormones,Particularlycorticosterone or cortisol are often used as an index of stress and any stimulus that causes an increase in HPA axis activity is identified as stressor (*Nagaraja et al., 2006)***.**

The stress response activates the SNS as well as the HPA axis. The SNS stimulates the adrenal glands to release (nor) epinephrine, which can be indirectly measured via salivary alpha-amylase (SAA) (*Nater and Rohleder, 2009*). Activation of the HPA axis leads to a release of CRH, adrenocorticotropic hormone, and glucocorticoids such as cortisol, the major stress hormones in humans. cortical readily enters the brain and modulates cortical as well as sub cortical structures involved in learning and memory, e.g. the amygdala, the hippocampus or the prefrontal cortex (*Merz et al., 2013; Wolf, 2009).*

The central control stations of the stress response are located in the hypothalamus and the brain stem. CRH neurons of the par ventricular nucleus initiate the stress response and comprise the principal hypothalamic regulator of the HPA axis. CRH stimulates the secretion

of ACTH from the anterior pituitary. Circulating ACTH acts on the zone fasciculate of the adrenal cortex where it stimulates the release of cortisol or corticosterone. In turn, cortisol feeds back to the brain to shut off further cortisol secretion. This negative feedback loop protects the organism from prolonged, detrimental cortisol exposure and keeps its concentration within a wide but stable operating range. The SAM originates in the locus ceruleus, and together with the HPA axis builds the effector limbs of the stress response. Cortisol stimulates hunger and feeding, and that adrenaline is part of the fight/flight response which shuts down digestion, we hypothesize that threat stress will stimulate eating more than challenge stress. One study comparing chronic physical stress (foot shock) *vs*Emotional stress in rats revealed that physical stress reduced consumption and preference for saccharin drinkcompared to water, whereas emotional stress increasedsaccharin preference and consumption compared to water.

Others found increased preference for palatable food with physical stress (foot shock), but only when rats were previously exposed to a history of dietary restriction. Repeated stressors in rats generally seem to reduce food intake (FI) and body weight (bw) (*Epel et al., 2007*). Oxidative stress contributes toward neuronal degeneration in the CNS in the process of aging as well as neurodegenerative diseases (*Hovatta et al., 2010*). The production of ROS is greatly increased under many conditions of toxic stress (*Liu & Schubert, 2009*). One of the reasons for stress-induced enhancement of free radicals may be the elevation of NO production (*Matsumoto et al., 1999*).

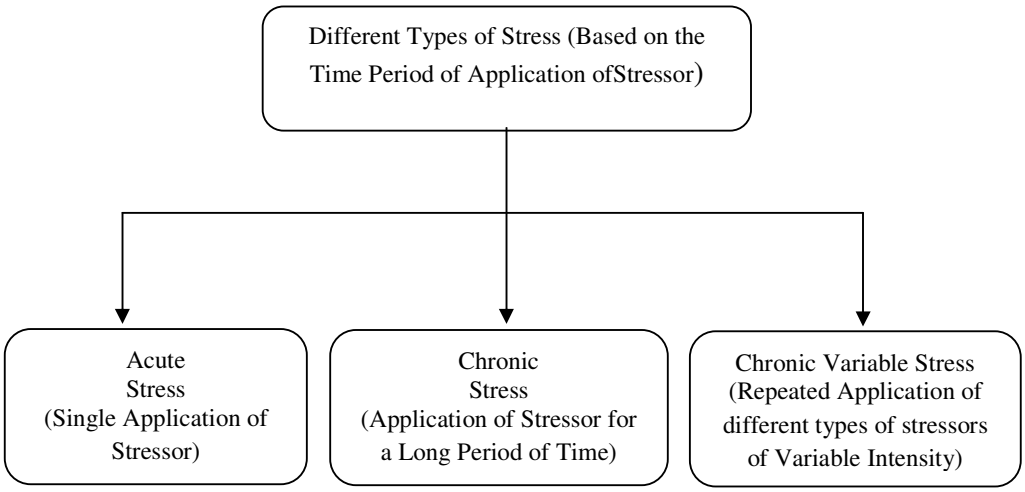
This is further supported by the present determination of Nitrite levels, which revealed significant increase in brain NO levels in stressed mice. The reactive nitrogen species along with ROS, working in concert with an inflammatory process, may play a substantial role in the pathogenesis of depression (*Matsumoto et al., 1999*). Stress has been shown to be responsible for the depletion of several free radical detoxifying enzymes such as glutathione peroxides, catalase, and superoxide dismutase (*Zaidi & Banu, 2004*).This results in oxidative burden, which has been implicated in stress as well as in the pathogenesis of several disease states. Since brain tissues consist of a high content of polyunsaturated fatty acids and one of the important consequences of oxidative stress is peroxidation of membrane lipids, this reaction produces marked damage to the structure and function of cell membranes in these tissues (*Jain et al., 1991*). Therefore, lipidperoxidation was supposed as the majorbiochemical alteration and consequence of oxidant-induced cell injury. Thus, the important consequences of stress could be attributed to stress-induced lipid peroxidation.

Everyone has their own coping strategies to handle stress, and some strategies are healthier than others. While some people are able to deal with stress without changing their normal behaviour, it is rare that stress does not have some adverse affects on an individual. Often times,thoseaffects reach the realm of eating behaviours. The investigation into whether stress leads to overconsumption or reduced food intake is widespread among different groups of humans and animals. Not only is the amount of intake examined, but also whether stress can invoke changes in the chosen types of food to eat. While there are clear biological changes that occur under stress, there are also emotional factors that can influence eating habits during stressful situations.

Whether the individual is a restrained or emotional eater can change the way food is used to cope with stress. With the increasing problem of obesity and the increased levels of stress born by individuals, the correlation between stress and eatingbehaviours may help in counteracting the obesity epidemic. Stress is a very crucial factor in the maintenance ofhealth and disease Stress induces changes in emotionalbehavior and anxiety like state, which are associated withoxidative damage, that is, free radical damage. Acuteimmobilization stress triggers numerous cellular cascadesthat lead to increase in ROS production. Because of thebrain high oxygenconsumption, abundant lipid content and relative paucity of antioxidant enzymes, the centralnervous system is highly vulnerable to free radical damage. Immobilization stress has also been reported to induce2-3-fold higher rise of plasma cortisol level;increasedcortisol level has been linked with anxiety-like behavior. It is been reported that stress triggers the motor alteration in different animal models and central nucleus of amygdale is important inmodulating affective response to stress. Natural productssuch as bioflavonoids possess very goodantioxidant proper and inhibit lipid per oxidation inbiologicalmembrane. Hesperidin is such natural bioflavonoidthat possesses very good antioxidant property and it has been proved to be very effective in various neurobehavioral diseases.

Antioxidants (AOs) are present in a variety of plants(including fruits, berries, and nuts) and promote health byremoving damaged free-radicals from the cellular environment,inhibiting the production of inflammatory mediators and blocking carcinogenic processes.The therapeutic application of a number of AOs in disease is currentlyunder investigation. Phloretin, present at high levels infruits, such as apples, pears, and strawberries, can exertanti-inflammatory and immunosuppressive effects on bothlymphoid- and myeloid-derived cells.

Developing an effective drug delivery system with the ability of crossing the blood brain barrier (BBB) is the crucial point intreating diseases of the Human central nervous system (CNS)effectively. Many potential drugs have been abandoned duringtheir development for their poor ability to cross the BBB insufficient quantities to produce a therapeutic effect. The BBB isnot only an anatomical barrier to the free movement of solutesbetween the blood and brain, but also a transport and metabolicbarrier. Consequently, developing tools and methods thatallow the therapeutic agents to delivery to the brain safely andeffectively in vivo is important.



ANIMAL MODELS COMMONLY USED

I. Physical stress models

Animal models of stress that use physical stress can be subdivided into:

1. Temperature fluctuation induced stress:
    a. Cold
    b. Heat
    c. Intense radiation
    d. Noise
    e. Pain (chemical & physical)
    f. Vibration and many others

II. Psychological stress models

Animal models of stress that use psychological stress can be subdivided into:

i. Neonatal isolation induced stress
ii. Predatory stress

iii. Day-night light change induced stress

iv. Noise induced stress.

III. Chronic unpredictable stress models

1) Stressors that challenge cardiovascular and metabolic homeostasis
2) Social stressors reflecting disturbed interaction among individuals

Different stressors of mild to moderate intensity are applied on variable basis so as to prevent the emergence of adaptation or resistance to one particular type of stressor. It involves the use of both physical and psychological stress models in a random way.

II. PHYSICAL STRESS MODELS

a. Temperature fluctuation induced stress

Acute change in temperature leads to stressful conditions by activation of temperature regulatory centre in the hypothalamus and subsequently HPA axis. It leads to acute release of adrenocortical hormones in the blood stream responsible for acute stressful response (*Sapolsky et al., 1986*). A sharp decrease in temperature using either cold water or freezer has been used frequently to induce acute stress.

i. Immersion in Cold Water (ICW)

In this method, the rats are placed individually in a tank of cold water (depth = 15.5 cm; temperature = 15-20°C) where they either swim or remain in an upright position, keeping their heads above water level (*Retana-Marquez et al., 2003; Iwona et al., 2003; Fernandez-Landiera, 2004; Yun et al., 2003*). This situation lasts for 15 minutes unless the rats sink. In that event, rats are removed before the cutoff time and are not included in the experiments. For acute stress, rats are sacrificed 30 minutes after the stress exposure. For chronic stress, animals are exposed to this stressor for 7-10 days. Rats are sacrificed 1 h after the last stress session. The major advantage of this type of stressor is that acute stress can be achieved in a relatively short period of time. However the major drawback of this model is that the body adapts to change in temperature on chronic exposure to low temperature and hence stress response gets highly diminished (*Pitman et al., 1988; Blustein et al., 1998*).

ii. Cold environment isolation

In this method, rats are individually kept in a freezer with a temperature maintained at 4°C. The rats are kept for 15 minutes once for acute stress and for 7-10 days to develop chronic stress (*Kvetnansky et al., 1971*). This sharp fall in temperature leads to a sharp increase in the level of adrenocorticoids, as explained above culminating in the development of stress response (*Kvetnansky et al., 2002; Staratakis&Chrousos, 1995*). Unlike the ICW model, rats

are prevented from drowning in cold water hence it is relatively safe model however it also suffers from same drawback of development of resistance/adaptation on chronic exposure.

iii.Immobilization induced stress

Immobilization has been used extensively as a stressor for the study of stress-related biological, biochemical and physiological responses in animals (*Kvetnansky&Mikulai, 1970; Kasuga et al., 1999; Marty et al., 1997*). Immobilization can be produced in two different ways. Animal can be either kept immobilized in a semi cylindrical acrylic tube (4.5 cm diameter and 12 cm long) with proper holes in it for air to pass (*Das et al., 2000*). Another way is to keep the animal with its limbs stretched on a board and its limbs are immobilized with adhesive tape. Movement of head is restricted by keeping the head in a metal loop coiled around the neck. The rats are kept immobilized in either of the above two ways for 150 minutes once to produce acute stress and for 7-10 days to produce chronic stress (*Dronjak&Gavrilovic, 2006*). The major advantage of using immobilization as a model of stress is that it produces an inescapable physical and mental stress to which adaptation is seldom exhibited (*Kasuga et al., 1999*).

iv. Electric foot shock induced stress

Electric foot shock (EFS) of mild intensity has also been used as a stressor. Rodents are very susceptible even to mild shock and exhibit rapid stress response. Researchers have used electric foot shock of varying degree to produce stressful conditions and hence to evaluate adaptogenic activity of various compounds. Stress by electric foot shock is given by placing the rats individually in a chamber with an electrified floor. Rats receive unavoidable electric foot shocks with an intensity of 3 mA, 200 ms of duration and a frequency of 1 per second over a 5-min period. For acute stress response, the rats are exposed once and sacrificed after 15 minutes of stress. Chronic stress is also produced by repeating the same treatment for 7-10 days and rats are sacrificed 1 h after the last stress session (*Retana-Marquez et al., 2003*). Some researchers have modified the method in which rats are subjected to inescapable electric foot shock for 60 minutes (0.15 mA shock, on a variable interval schedule with a mean inter shock interval of 60 seconds) (*Taysse et al., 2005*). The biggest advantage of this model is that it effectively produces high degree of stress in the animal. The major disadvantage of this model is the hazard of electric shock causing death of the animal and special caution that is required to perform this methodology.

v. Forced swimming induced stress

It is the tendency of the living being to escape or avoid a noxious stimuli/condition. If the animal is not able to escape the stressful stimuli or it feels threatened, the animal will show stress response. This principle is used for developing forced swimming model for inducing stress in laboratory animals. In order to produce swimming induced stress, rats are made to swim in a cylinder (30 cm diameter and filled to a height of 20 cm with 15 cm of space above the head of the rat) for a single session of 2 h duration for acute stress, or for one 2 h session a day for five consecutive days for chronic stress (*Ferry et al., 1991*). Some authors have used forced swimming in warm (20°C) water for 3 minutes with the total session lasting for 1 h (*Kitchen & Pinker, 1990*). Although forced swimming induced stress is a highly safe model, adaptation to chronic swimming induced stress has been reported and inter-strain differences between rats to forced swimming behavior have also been documented (*Armario et al., 1995*).

III. PSYCOLOGICAL STRESS MODELS

a. Neonatal isolation stress

Early life events have profound consequences on subsequent quality of life. It has been shown that the early life stress of neonatal isolation in rats has immediate and enduring neural and behavioral effects (*Kuhn et al., 1990*). Such effects may reflect, in part, stress-induced morphological changes in hippocampus and other brain regions (*Kosten et al., 2005b*). In fact, the hippocampus provides negative feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis (*Herman &Cullinan, 1997*) and hence neonatal isolation induced stress can represent the stress response that may lead to neuro-degeneration at an early stage of life. This stress procedure is also useful in evaluating the effect of stress on cognition and memory development. In the neonatal isolation procedure, the litter of the inbred strain is removed from the cage on second day after the birth, weighed and placed individually in an opaque plastic container (9 cm diameter and 8 cm deep) with no bedding for 1 h (between 09:00 and 12:00) in a heated (30°C), humidity controlled chamber with white noise to mask other pups calls. The chamber has to be located in a room separate from animal colony facility. Containers are placed 20-30 cm apart. After 1 h period the litters are placed back with their dams in home cage (*Kosten et al., 2000; Kosten et al., 2004*). This isolation procedure continues up to 8 days and hence it is used to induce chronic stress only. Neonatal isolation stress model has be used extensively to demonstrate the effect of early lifetime stress on vulnerability to addiction (*Kosten et al., 2005a*), and response to psychostimulants by impairment of hippocampal-dependent context induced fear in adult male rats.

b. Predatory stress

Direct encounter of an animal with its natural predator is one of the most stressful and anxiogenic event it can face and it leads to rapid development of „flight or fight" response (*Lupien et al., 2006*). Exposure of rodents to natural predators or to their odors may induce stress like states (*Adamec& Shallow, 1993*). Under such circumstances, there is rapid sympathetic activation leading to rise in the levels of adrenocorticoids in blood causing acute stress response to develop. Direct encounter with a predator has been effectively used to evaluate the biochemical and physiological changes produced during such stressful conditions (*Marilia et al., 2007*). Predatory stress in mice is induced by series of short exposures to natural predator like cat (*Blanchard & Blanchard, 1989*) or to any substance having the odor of cat like the fecal pellets of cat (*Berton et al., 1998*). In one of the methods, mice are placed individually in different cages and after four initial 20-min cage habituation sessions each subject is submitted to two randomly-assigned 20-min predator confrontation sessions. Change in behavioral pattern such as locomotion, shrieking like voices and endocrinological changes after the stress exposure are observed (*Blanchard et al., 1998*). Another free-exploration test (*Griebel et al., 1993*) was used consists of a PVC box (30x20x20 cm) covered with Plexiglas and subdivided into six equal square exploratory units, which are all interconnected by small entries. It could be divided in half lengthwise by closing three temporary partitions. Approximately 20 h before cat exposure, each subject is placed in one half of the apparatus with the temporary partitions in place, in order to be familiarized with it. The floor of this half was covered with fresh sawdust and the animal is given unlimited access to food and water. On the test day, mice of each strain are randomly allocated to the following four groups.

(a) Naive clay: animals are exposed to both familiar and novel compartments by removal of the temporary partitions. The novel compartment contains three modeling odor-free clay pellets.

(b) Naive feces: animals are exposed to both familiar and novel compartments. The novel compartment contains three cat feces pellets.

(c) Exposed clay: subjects are removed from the free-exploration box and confronted individually with a cat during a 5 min session. The cat cage consists of a PVC box (82x56x62 cm) subdivided into two compartments, one containing the cat, the other the mouse. Separation consists of a transparent PVC wall with holes allowing the cat to reach the other side with its paws. The mouse is then put back in the free-exploration apparatus and is

exposed 1 h later to both familiar and novel compartments. The novel compartment contains three modeling odor-free clay pellets.

(d) Exposed feces: same as previous group, but the novel compartment contains three pellets of feces from the cat used during exposure. The behavior of the mouse is observed under red light for 5 min via a closed circuit TV camera by an observer located in an adjacent room.

The following parameters are recorded:
(a) Time spent in the novel compartment; (b) total unit entries and (c) total number of rearings.

The results are expressed as mean percentage of time spent in the novel compartment, mean total number of novel unit changes, and mean total number of rearings. Marmosets (*Callithrixpenicillata*) have also been employed for induction of predatory stress in a test battery known as Marmoset Predator Confrontation Test [MPCT] (*Cilia & Piper, 1997*). This model compares the behavioral response of experienced versus naïve adult black tufted-ear marmosets in confrontation with a taxidermized wild-cat predator stimulus. After four initial 20-min cage habituation sessions, each subject is submitted to two randomly-assigned 20-min predator confrontation sessions. Confrontation with the predator induces significant behavioral changes; i.e., proximic avoidance and *tsik-tsik*alarm call. Anti-stress drug administration, concomitant to predator exposure, reverses the behavioral changes observed (*Barros et al., 2004*). Predator induced stress is an established model to induce short term acute stress response but its major disadvantage is development of habituation to predator exposure hence the use of this model for inducing stress is justified for developing only acute stress.

c. Day-night light change induced stress

Changes in the circadian rhythm have profound effect on physical and psychological well being of an individual (*Atcheson& Tyler, 1975*). Laboratory animals, when subjected to abrupt changes in day-night light pattern, exhibit acute stress response (*Kosten et al., 2005b*). Changes in circadian rhythms are regulated by pineal gland through the secretion of melatonin (*Nicholsonet al., 1985*). Melatonin is released from the pineal gland in response to dark or dim light where as its functional antagonist serotonin is secreted in response to bright light. It is this serotonin-melatonin cycle that is responsible for regulation of sleep-awake state of the body (*Bermudez et al., 1983; Hamm et al., 1983)*. To induce stress, cages of rat or mice are kept under bright light from 19:00 h over night (in the dark phase) and cages are

kept in dark room with no light from 12:00 h in the light phase for 180 minutes for 7-10 days (*Marcelo et al., 2007*). This method is suitable for inducing short term stress response. Generation of stress can be evaluated by measuring the biochemical parameters associated with chronic stress response (*Rai et al., 2003*). The major disadvantage of this model is that it can be effectively used to generate short term stress response as on repeated exposure to this type of stressor, the animal adapts to the changed day-night light pattern. This major drawback can be minimized by using this model as a part of chronic unpredictable stress protocol.

d. Noise induced stress

Noise as a stressful stimulus is a widely accepted fact. A large number of people are exposed to potentially hazardous levels of noise levels in daily modern life. Experimental studies have demonstrated ultra structural modifications in rat cardiomyocytes mainly in mitochondria due to noise stress. These subcellular alterations are related to an imbalance in calcium homeostasis, which is supposed to be sustained by increased catecholamine innervations (*Paparelli et al., 1992*). When noise exposure of any kind exceeds 90 dB, noise becomes a stressor (*Ramsey, 1982*). Noise stress has a depletory effect on free radical scavenging enzymes in the brain leading to moderate to severe oxidative stress (*Samson et al., 2005*) which can be a potential basis for hearing loss (*Fechter, 2005*). Noise stress in laboratory rats can be produced by loudspeakers (15 W), driven by a white noise generator (0-26 kHz), installed 30 cm above the cage. Thus a noise level can be set at 100 dB or above uniformly throughout the cage and can be monitored by a sound level meter. Each animal to be treated is exposed to noise stress for 4 h/day for 15 days. Control group rats are also kept in the above described cage during the corresponding period of time, without noise stimulation to avoid the influence of handling stress on evaluation of effects due to noise exposure (*Ravindran et al., 2005; Manikandan& Devi, 2005*). The effect of noise stress exposure can be determined by estimating the brain biogenic amine level.

iv. Chronic variable (unpredictable) stress models

The major disadvantage of both physical stress models and psychological stress models is the development of adaptation / resistance on chronic exposure. The changes in physiological and behavioral responses to chronic stress can be related to the adaptation of the HPA axis. When the same stressor is repeated, the HPA response undergoes desensitization or become stable as it has been reported that rodents repeatedly exposed to restraint stress exhibited a habituated corticosterone response, when they were subsequently challenged with an acute

exposure to restraint (*Magarinos& Evans, 1995; Gadek-Michalska&Bugajski, 2003*). On the other hand, the exposure to a multiple stress paradigm produced continued elevation in corticosterone levels, when the animals were subsequently subjected to acute restraint stress (*Magarinos& Evans, 1995*). It has also been suggested that the adaptations of HPA axis depend on type, duration and severity of the stress regime (*Blanchard et al., 1998; Gadek-Michalska&Bugajski, 2003*). To prevent the development of resistance, Chronic Unpredictable Stress (CUS) models have been developed which involve the use of various physical and psychological stressors in a predetermined manner so that the animal is not able to adapt to the stressor. Adaptation to one type of stressor has been effectively prevented by employing various stressors such as immobilization stressor for 15 minutes followed by overnight sleep deprivation and rotation of the cage at a predetermined speed (horizontal shakes at high speed) for 50 minutes followed by swim stress in water (20ºC) of 4 minutes (*Ortiz et al., 1996*). Wetting the saw dust bedding of the animal all day to restrict movement followed by electric foot shock (ten shocks of one second duration each, in an unpredictable manner, at the intensity level of 0.4-1.8 mA) and stroboscopic light (for 13 h, 10 Hz) has also been used as a part of CUS protocol (*Margus et al., 2007*).

Some researchers have used exposure to predator odor induced stress as a part of CUS protocol, in which mice are placed in a novel cage containing cat litter soiled with cat feces and urine (*Anisman et al., 2007*). Various authors have modified the stress models in order to accommodate them in their respective CUS protocol. Other additional stressors that have been applied as a part of CUS protocol are tail pinch with a clothes-pin placed 1 cm distal from the base of the tail for 5 min, strong illumination during predicted dark phase for 12 h, movement restriction in a small cage (11 cm x16 cm x 7 cm) for 2 h (*Ortiz et al., 1996*), ether anaesthesia until loss of reflex (*Renard et al., 2005*), and subcutaneous 0.9% saline injection (*Ladd et al., 2004*). Chronic variable stress models have been proven to be more useful as they are devoid of the problem of resistance in the animal species towards the commonly used stressors and also have the advantage of the development of effective and long-term stress response. Thus CUS models are nowadays the preferred models for generation of a stress response.

Stress has been postulated to be involved in the etiopathogenesis of a variety of disease state including hypertension, coronary heart disease (Roy et al., 2001), gastric ulcers (*Yadin& Thomas, 1996*), diabetes (*Fitzpatrick et al., 1992*), immunosuppression (*Purret, 2001*), mental depression, memory loss (*Gareri et al., 2000*), and host of other diseases.

v. Common Motifs in Neurodegeneration

Neurodegenerative disorders such as AD and PD account for a significant and increasing proportion of morbidity and mortality in the developed world (*Hebert et al., 2001*). Largely as a result of increased life expectancy and changing population demographics (i.e., the aging of baby boomers), neurodegenerative dementias and neurodegenerative movement disorders are becoming more common (*Brookmeyer et al., 1998; Samii et al., 2004*). As our population ages, an improved understanding of these diseases will be vital to developing more effective therapies and combating the staggering personal, social, and economic costs of these diseases (*Ernst et al., 1997*). Unifying theories of pathogenesis in neurodegenerative disease provide an avenue for developing therapeutic strategies with broad applicability for disease prevention and an opportunity for decreasing morbidity and mortality from these disorders in the elderly population (*Forman et al., 2004*). Converging lines of investigation have revealed a potential single common pathogenic mechanism underlying many diverse neurodegenerative disorders.

v.a.Mechanisms of Neuronal Death

Acute injury to cells causes them to undergo necrosis, recognized pathologically by cell swelling, vacuolization and lysis, and associated with calcium (Ca^{2+}) overload of the cells and membrane damage. Necrotic cells typically spill their contents into the surrounding tissue, evoking an inflammatory response. Cells can also die by apoptosis or programmed cell death, a slower process that occurs normally during development and is essential for many processes throughout life, for example development, immune regulation and tissue remodeling.Apoptosis, as well as necrosis, occurs in many neurodegenerative disorders including acute conditions such as stroke and head injury (*Bredesen, 1995*). The distinction between necrosis and apoptosis as processes leading to neurodegeneration is not absolute, for challenges such as excitotoxicity and oxidative stress may be enough to kill cells directly by necrosis, or, if less intense, may induce them to undergo apoptosis. Both processes, therefore, represent possible targets for putative neuroprotective drug therapy.

Pharmacological interference with the apoptotic pathway may become possible in the future, but for the present, most efforts are directed at the processes involved in cell necrosis, and at compensating pharmacologically for the neuronal loss.In recent years there has been an increasing interest in the studies on Neurodegeneration, including the physiological or programmed neuronal death and the cell disruption occurring as a consequence of necrosis. This interest has been greatly stimulated by the fact that precipitation and localization of

neuronal destruction is a central event in the course of many acute and chronic disorders of the CNS. These disorders include stroke (anoxia-ischemia), hypoglycemia, cerebral trauma, epilepsy and several devastating neurodegenerative diseases, such as ALS, PD, AD and HD.

Among the cellular mechanisms possibly involved in neuronal death in neurodegenerative disorders, three closely related factors seem to play important roles: (1) the generation of reactive oxygen species (ROS) or free radicals, (2) the over activation of synaptic excitatory amino acid (EAA) receptors, and (3) the increase in cytoplasm free Ca^{2+} concentration and (4) infection. As shown in Fig. 1, the links between these factors are multiple and an initial event may lead, in a cascade manner, to the generation of further alterations. In addition to these, several other factors like selective vulnerability and exquisite specificity of the disease process for particular types of neurons; genetic predisposition; excitotoxicity; oxidative stress; environmental triggers – infectious agents, toxins, brain injury; aging and disruption in energy metabolism (*Standaert et al., 2009*).

v.b.Oxidative Stress

Oxidative stress means an unbalance between pro-oxidants and antioxidant mechanisms. This results in excessive oxidative metabolism. This stress can be due to several environmental factors such as exposure to pollutants, alcohol, medication infections, poor diet, toxins, radiation etc. Oxidative damage to DNA, proteins, and other macromolecules may lead to a wide range of human diseases most notably heart disease and cancer. Free radicals are capable of attacking the healthy cells of the body by oxidative stress. This may lead to damage, disease and severe disorders. Cell damage caused by free radicals appears to be a major contributor to aging and diseases like cancer, heart disease, decline in brain function, decline in immune system etc.

v.c. Lipopolysaccharide

Lipopolysaccharide (LPS), an endotoxin of the gram-negative bacteria cell wall, is the major cause of sepsis. Endotoxins play a major role in developing stress and has a cascading effect on the functioning of the defense mechanisms, neuroendocrine, neurotransmitter, behavior, cognition etc.,

Exposure to stressful events like infection, often results in long-lasting changes in the response of a variety of systems. For example, repeated exposure to the same stressor (homotypic stress) often results in habituation of the hypothalamic–pituitary–adrenal (HPA) axis and brain stem catecholaminergic activity. Conversely, exposure to a test stressor that is different (heterotypic stress) from that used during the initial repeated exposure results in

sensitization of the HPA axis and brain stem catecholaminergic activity. In addition, a single exposure to a stressor has been shown to sensitize central pathways involved in drug reward and neuroendocrine responses (*van Dijken et al., 1993*).LPS is one of the most common inflammogens used to investigate the impact of inflammation on neuron death. The production of intracellular ROS results from the process of normal cellular function and metabolism and may originate from multiple cellular sources, such as xanthine oxidase, mitochondrial electron transport, NADPH oxidase, peroxisomes, and the endoplasmic reticulum. The identification of ROS as a primary factor in LPS-mediated neurodegeneration, the localization of the source of ROS causing the neurotoxin effect and the elucidation of the signaling Pathway driving microglia over-Activation is of paramount importance to understanding the molecular mechanisms of the neurodegenerative disease state. The brain is particularly susceptible to the effects of ROS due to its high consumption of oxygen and modest antioxidant defenses. These features are coupled with high concentrations of polyunsaturated fatty acids (PUFA) which are easily oxidized and known to generate oxygen radicals following an insult *(Rice-Evans & Burdon, 1993).*

vi. Herbal Medicines

In traditional practices of medicine, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases such as AD and other memory related disorders. Various studies have been undergone to identifying potential new drugs from plant sources, including those for memory disorders. There are numerous drugs available in market that have been isolated from plants, e.g. alkaloids from plant sources have been investigated for their potential in AD therapy, and are now in clinical use. Usually herbal preparations are well tolerated but they may have harmful side-effects, including interactions with pharmaceuticals (*Howes& Houghton., 2003*). Herbal medicines, such as, *Ginkgo biloba*, *Bacopamonier* (Bramhi) (*Das et al., 2002*), Shankhpushpi, etc, has been found to increase memory power.

vi.a Herbal Medicine as Antioxidants

Antioxidants are man-made or natural substances that may prevent or delay some types of cell damage. Antioxidants are found in many foods, including fruits and vegetables. They are also available as dietary supplements. Some of the antioxidants found in natural substances are beta-carotene, lutein, lycopene, selenium, vitamin A, vitamin C, vitamin E etc., vegetables and fruits are rich sources of antioxidants. There is good evidence that eating a diet with lots of vegetables and fruits is healthy and lowers risks of certain diseases. But it

isn't clear whether this is because of the antioxidants, something else in the foods, or other factors.High-dose supplements of antioxidants may be linked to health risks in some cases. For example, high doses of beta-carotene may increase the risk of lung cancer in smokers. High doses of vitamin E may Increase risks of prostate cancer and one type of stroke.

Antioxidant supplements may also interact with some medicines. To minimize risk, tell you of your health care providers about any antioxidants you use. All living organisms utilize oxygen to metabolize and use the dietary nutrients in order to produce energy for survival oxygen thus is a vital component for living. Oxygen meditates chemical reactions that metabolize fats, proteins, and carbohydrates to produce energy. While oxygen is one of the most essential components for living, it is also a double edged sword. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals. Apart from diet, the body also has several antioxidant mechanisms that can protect itself from ROS mediated damage. The antioxidant enzymes – glutathione peroxidase, catalase, and superoxide dismutase are such enzymes. They require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for their activity. It has been suggested that an inadequate dietary intake of these trace minerals may also lead to low antioxidant activity.

vi.b. Phytochemicals

As human life expectancy has increased, so too has the incidence of stress related neurodegenerative disorders such as AD, PD and HD. Phytochemicals, especially flavonoids have a wide range of medicinal actions, and throughout history, they have been used to treat many different types of diseases. In the treatment of many diseases, antioxidant therapy plays a key role, so current research is now directed towards finding naturally occurring antioxidant of plant origin.Photochemical are non-nutritive plant chemicals that have protective or disease preventive properties.

It is well-known that plants produce these chemicals to protect themselves but recent researches demonstrate that they can also protect humans against diseases *(Viswanatha et al., 2012)* some of the well-known phytochemicals are flavonoids in citrus fruits. In Indian system of medicine, phytochemicals are being used to alleviate, ameliorate and/or treat many illnesses successfully. The flavonoids are also used as a nutritional supplement for any ailments. Hesperidin, a flavone glycoside copiously found in peels of sweet orange and lemon, is an inexpensive by-product of citrus cultivation.

Hesperidin is effectively used as a supplemental agent andit has been reported to possess significant anti-inflammatory, analgesic, antifungal, antiviral antioxidant, and anticancer activities *(Vaibav et al, 2010).*But there is lack of studies related to neuroprotective effect of hesperidin in (LPS)-induced endotoxicNeurodegeneration. The general behaviour, anxiolytic and antidepressant activity along with antioxidant enzyme and non-enzyme status will be analysed in rat brain,along with the pattern of food and water intake for the first time to assess the protective effect of hesperidin against endotoxin-induced Neurodegeneration. We anticipate that the behavioural tests used in the present study could contribute to the evaluation of hesperidin against endotoxin-induced neurodegeneration and may shed an insight into the mechanism of action. Hence, a special attention is focused to understand the treatment of neurodegenerative diseases by natural flavonones, preferably hesperidin.There are, indeed, a multitude of paradigms assessing various aspects of the behavioral performance like anxiety and depression, changes in feeding pattern, body mass and biochemical analysis. Till now, some of the paradigms have not been used at all in the evaluation of hesperidin against behavioral consequences of adult rats when challenged with LPS.Hence, in order to contribute further to the knowledge of therapeutic effect of hesperidin, and its rich abundance, the objective of the present study is to subject hesperidin to test against endotoxin-induced neurodegeneration and in rat models.

vi.c. Flavonoids

Over the last decade, renewed interest has been shown toward these flavonoid compounds, with some 4000 having been isolated so far. Only a handfulhas been extensively studied for their medicinal properties (*Rice-Evans, 2004*). In the plant kingdom, they function as special pigments designed to prevent sunlight and toxin-induced free radical damage.Recent studies have shown some rather remarkable medicinal properties of flavonoid compounds. For example, some havebeen shown to have anticancer properties, antiviral and antibacterial activity (*Hirano, 1989*), and immune stimulating qualities (*Wacker&Hilbig, 1978*), as well as, offering protection against strokes and heart attacks (*Stimpel et al., 1984; Hertog et al., 1993; Krieglstein et al., 1986*).The medicinal properties of plants have been investigated in the light of recent scientific developments throughout the world, due to their potent Pharmacological activities, low toxicity, and economic viability. Ayurveda has a clinical specialty called *Rasayana*, which prevents diseases and counteracts the ageing process by means of optimization of homeostasis. It has been reported that the *Rsayanas* are rejuvenators, nutritional supplements, and strong antioxidants (*ShanmugaSundaram &*

Gowtham, 2012).It is estimated that nearly 25% of modern drugs directly or indirectly originated from plants (*De Smet, 2002*). There are more than 120 traditional medicines in use for the therapy of CNS disorders in Asian countries. Some of their therapeutic effects have been confirmed by recent clinical studies. An ethnopharmacological approach has provided a potentially rich source for drug discovery and development (*Harvey, 1999*).

Many drugs currently available in Western medicine were originally isolated from plants. Although a large number of compounds have been isolated, most of these resources have not yet been fully characterized for pharmacological purposes.Although some plants have shown beneficial effects, further studies regarding the compounds responsible for activity are necessary to identify which compounds are responsible for the pharmacological activities observed, or if compounds act synergistically to enhance activity. For many plants and compounds that have shown activities relevant to therapy, clinical data are very limited. Clinical efficacy and potential toxicity of active plants and compounds in larger trials require further assessment before recommendations regarding their use can be established. Currently, drug research focuses on mechanism based drug development and understanding the etiology of the disease. Although advances in the symptomatic therapy of the neurodegenerative disorders have improved the lives of many patients, the drugs still have some drawbacks (*Melanie-Jayne & Peter, 2003*).

There has been a recent explosion of interest by research scientists in the flavonoid compounds, with a multitude of medically useful properties having been demonstrated in experimental, as well as, clinical studies of flavonoids. For instance, flavonoids have been shown to act as powerful free radical scavengers for multitude of free radical species, even the powerful proxynitrite radical. In addition, several flavonoids have shown powerful metal-chelating properties, especially for iron and copper, two of the most potent-free radical catalysts (*Jovanovic, 1998*). Of equal Importance are several studies that have shown that flavonoids interact with cell membranes, improving their fluidity, thereby protecting them from lipid peroxidation (*Morel et al., 1998*). Along these same lines is the protection of microvessels in the nervous system by specific flavonoids from free radical damage (*Saija et al., 1995; Ratty & Das, 1988*). This not only prevents leakage of such vessels, but has been shown to preserve the blood–brain barrier as well (*Kuttan et al., 1981*). There is also evidence that several of the flavonoids can inhibit platelet adhesiveness, thereby preventing strokes

(*Robert et al., 1977*). Finally, some of the flavonoids have the unique ability to inhibit certain enzymes, such as the COX-2 enzyme (*Tzeng et al., 1991; Kim et al., 1998*).

vi.d. Flavonoids and Inflammation

Neuroinflammation plays a vital role in neurodegenerative diseases and its inhibition by neuroprotective herbs, the antioxidant activity of herbal extracts is certainly another important aspect of neuro protection. A variety of herbal extracts and their components have been demonstrated to exert neuroprotective effects associated with antioxidant activities, either by directly stimulating antioxidant response genes or by potentiating the bodies' own natural antioxidant defense systems (*Fahn& Cohen, 1992; Metodiewa&Koska, 2003*). This is supported by the findings that many herbal extracts and their components with neuroprotective activities exert both antiinflammatory and antioxidant effects at the same time (*Ahlemeyer&Krieglstein, 2003; Kim et al., 1998; Oyama et al., 1996*). One of the more useful Properties of flavonoids is their ability to prevent inflammation via their interaction, with various steps, along the eicosanoid pathway.

For example, certain flavonoids, such as the flavones and hesperidin, in high concentrations, can directly inhibit the release of arachidonic acid from the cell membrane. Others, such as quercetin, Myricetin, kaempferol, naringenin, and hesperidin, can inhibit activation of phospholipase A2, which initiates the release of arachidonic acid from the cellmembrane.Certain flavonoids have been shown to either inhibit lipoxygenase (LPO) (hesperidin) or the cyclo-oxygenase-2COX)-2] enzymes, or even both (quercetin) (*Lindah l& Tagesson, 1997*). Pycnogenol is known to inhibit LPO, but not COX (*Pietta, 1998*).There is at least some evidence that prostaglandins can inhibit glutamate uptake, thereby increasing neurodegenerative excitotoxicity(*Rohdewald, 1998*).Knowing that Alzheimer's patients have elevated levels of eicosanoids prostaglandin D2 (PGD2) and thromboxane-B2 (TXB2), it seems reasonable that flavonoids that inhibit the enzymes known to contribute to this abnormal rise in inflammatory substrates, should at least slow the progress of neurodegeneration or even prevent it. This appears to be the case in the clinical studies cited earlier in the text.

vi.e. Flavonoids as Free Radical Scavengers

The flavonoid compounds have two properties that make them especially useful as antioxidants. First, many are powerful, primary free radical scavengers against a wide variety of radicals, including singlet oxygen, superoxide, peroxyl, hydroxyl, and the peroxynitrite

radicals. Second, several are known to be very effective metal chelators (*Saija et al., 1995*). Most flavonoids are present in plants as glycosides (*Duthie et al., 1997*). In the intestines, this moiety is cleaved-off, leaving the aglycone form of the flavonoid. It is the aglycone form that is thought to have the highest antioxidant activity in biological systems (*Griffiths, 1982*).

There is experimental evidence that hydrogen peroxide accumulation occurs during the process of catecholamine catabolism, making it especially important in PD. Recent evidence also indicates that hydrogen peroxide (H_2O_2) plays an important role in the toxicity of Alzheimer's plaques. As we have seen, iron accumulation within neurons is characteristic of ageing of the nervous system, but is especially high in the case of Neurodegeneration. A multitude of phytochemicals have specific properties that make them especially useful in combating neurodegeneration, and a list of nutrients that stimulate energy generation, primarily through the mitochondrial system are Coenzyme Q10, acetyl L-carnitine, α‑lipoic acid, vitamin K, nicotinamide, riboflavin, pyridoxine, folate/B12, thiamine and magnesium.

vi.f. Nutraceuticals

Nutraceutical, a portmanteau the words "nutrition" and "pharmaceutical", is a food or food product that provides health and medical benefits, including the prevention and treatment of disease.Health Canada defines the term as, "A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease."

Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered foods, herbal products, and processed foods such as cereals, soups, and beverages

With recent developments in cellular-level nutraceutical agents, researchers, and medical practitioners are developing templates for integrating and assessing information from clinical studies on complementary and alternative therapies into responsible medical practice.

The term nutraceutical was originally defined by Dr. Stephen L. DeFelice, founder and chairman of the Foundation of Innovation Medicine (FIM), Crawford, New Jersey. Since the term was coined by Dr. DeFelice, its meaning has been modified by Health Canada which defines nutraceutical as a product isolated or purified from foods, and generally sold in

medicinal forms not usually associated with food and demonstrated to have a physiological benefit or provide protection against chronic disease.

Examples: beta-carotene, lycopene. Nutraceutical foods are not subject to the same testing and regulations as pharmaceutical drugs. The following is an incomplete list of foods with reported medicinal value:

- ***Antioxidants***: resveratrol from red grape products; flavonoids inside citrus, tea, wine, and dark chocolate foods; anthocyanins found in berries
- ***Reducing hypercholesterolemia***: soluble dietary fiber products, such as psyllium seed husk
- ***Cancer prevention***: broccoli (sulforaphane), fiddleheads (*Matteucciastruthiopteus*)
- ***Improved arterial health***: soy or clover (isoflavonoids)
- ***Lowered risk of cardiovascular disease***: α- linolenic acid from flax or seeds. In addition, many botanical and herbal extracts such as ginseng, garlic oil, etc., have been developed as nutraceuticals. Nutraceuticals are often used in nutrient premixes or nutrient systems in the food and pharmaceutical industries.

vi.g. Hesperidin

Hesperidin is a flavonoid fount in the rinds of citrus fruits. Flavonoids are type of polyphenol, which are antioxidant that gives citrus fruit their color and Taste. It is also sold as a health supplement to repair and prevent cardiovascular disease and also hesperidin is a flavonone glycoside fount in citrus fruits. Its name is derived from the word “hesperidium” for fruit produced by citrus trees. Hesperidin was first isolated in 1828 by French chemist from the white inner layer of citrus peels. Hesperidin is a plant chemical that is classified as a “bioflavonoid” and people use it is a medicine.Citrus fruits and their products are important sources of health-promoting constituents and are widely consumed around the world (*Benavente& Castillo, 1997*). Hesperidin is a naturally occurring flavonone that exists in citrus and other plants and can be isolated in large amounts from the peels of *Citrus aurantium*(bitter orange), *Citrus sinensis*(sweet orange), and *Citrus unshiu*(satsuma mandarin) (*Crozier et al., 2009*).Hesperidin is reported to exert a wide range of pharmacological effects such as antioxidant, antiinflammatory, antihypercholesterolemic and anticarcinogenic properties (*Chen et al., 2010*). It has also been demonstrated that hesperidin can protect neurons against various types of insults associated with many neurodegenerative diseases (*Cho, 2006*).

Hesperidin is a compound in orange peels that gives the flavonoid hesperidin to the body, and this flavonoid mediates most benefits of hesperidin including an Increase in circulation and possible brain protective effects. Hesperidin, alongsidearningenin, is known as the main citrus flavonoids. That being said, in animal studies oral intake of hesperidin at a dose similar to that used in humans seems to be a very potent cardioprotection agent and is quite protective of the brain in responseto variousstressors; the protection is antioxidative in nature, but it seems to work through a currently not identified antioxidant responses from the genome. Aside from the protective effects (most notable in the heart and brain, but extend to every organ), hesperidin may be able to reduce a lack of appetite and have minor anti-allergic properties.

Hesperidin is also known as 5,7,3'-trihydroxy-4'-methoxyflavanone, hesperitin-7-O-rutinoside, hesperitin glycoside, glucosyl hesperidin, vitamin P, hesperitin and G-hesperidin. Hesperidin can be found in (measured in aglycone equivalents, or in other words measuring hesperitin, unless otherwise specified): Commercial sweet orange (*Citrus sinensis*) juiceproducts in the range of 12.7-15.9 mg per 100 mL (*Mazzaferro&Brecci, 2012*) and around 15.25+/-8.21 mg per 100 g fresh fruit weight (*Julia et al., 2006; Berhow, 1856*).

Our orange (Usually *Citrus aurantium*, also refers to bergamia and myrtifolia) tend to be high in flavanones at 47mg/100g fresh weight, although they tend to be neohesperidin and naringin (narirutin) rather than hesperidin (*Julia et al., 2006*) and contain barely any detectable hesperidin (at highest, 4.7µg/100g fruit weight) (*Berhow, 1856; Russell et al., 1987*). Tangerines or 'Mandarin Oranges' (*Citrus reticulata*) at 19.26+/-11.56mg/100g fresh fruit weight (*Julia et al., 2006; Berhow, 1856; Wilfred & Christ, 1997*) and the sundried peel (known as Chenpi in Traditional chinese medicine, or as *CitriReticulataePericarpum*) contain hesperidin at 50-100 mg/g.Tangors, which are orange and tangerine hybrids, at 15.42+/-7.00mg/100g fresh fruit weight (*Julia et al., 2006; Berhow, 1856; Wilfred & Christ, 1997*). Tangelos,which are tangerine hybrids with grapefruit (*Citrus paradisi*) or pumello (*Citrus grandis*) at 4.21+/-2.93 mg/100 g fresh fruit weight (these fruits are highest in neohesperidin) (*Julia et al., 2006; Berhow, 1856; Russell et al, 1987*). The peels of tangerines, however, appear to have 5-10% of their weight as hesperidin after 5-7 Days of drying (to remove water content and concentrate the hesperidin) and as such a 500 mg supplemental dose of hesperidin can be achieved by 5-10 g of the dried tangerine peel. This is a low cost alternate

assuming that the peel is thoroughly scrubbed prior to drying to remove possible contamination and grime collected on the peel.

vi.h. Antioxidant Properties of Hesperidin

The antioxidant hesperidin, a major flavonoid in sweet orange and lemon, was evaluated using chemical and biological systems hesperidin treated with stressing agent's hydrogen peroxide or paraquat. Hesperidin was able to reduce significantly the level of the free radical $DPPH^{\cdot}$ with similar efficacy of trolox (positive control *(Patricia et al., 2005*) When the yeast cells were exposed to the flavonoid hesperidin before the stressing agents, there was a significant increase in the survival of all strains. Paraquat induced higher catalase and superoxide dismutase than did H_2O_2, which only increased catalase activity. Previous addition of hesperidin to these treatments was able to reduce significantly both enzymatic levels. These observations clearly demonstrate that hesperidin provides strong cellular antioxidant protection against the damaging effects induced by paraquat and peroxide hydrogen. Antioxidants are vital substances that protect the body from damage caused by free radical-induced oxidative stress.

Free radicals can be generated from metabolic pathways within body tissues and can also be introduced from external sources such as drugs, food, UV radiation and environmental pollution (*Jungsook, 2006*). Free radicals have been implicated in the cause of several diseases such as liver cirrhosis, atherosclerosis, cancer and diabetes and play an important role in ageing. Oxidative stress can also contribute to the development of neurodegenerative disorders, such as AD and PD as well as other diseases.

These free radicals attack unsaturated fatty acids of biomembranes, resulting in lipid peroxidation and desaturation of proteins and DNA, causing a series of deteriorative changes in thebiological systems leading to cell inactivation. Thus, antioxidants are important inhibitors of lipid peroxidation, not only for food protection but also in defending living cells against oxidative damage. Lipid peroxidation is an important deteriorating reaction in food during storage and processing that causes a loss in nutritional quality.

The addition of antioxidants is required to preserve food quality. Antioxidant supplements orantioxidant-rich food is used to help the human body reduce Oxidative damage from Free radicals and active oxygen species. Another synthetic antioxidants such as butylated hydroxyl toluene (BHT),butylated hydroxyl anisole (BHA) and trolox are widely used as antioxidants in the pharmaceutical and food industry. However, they have been shown to have toxic

and/or mutagenic effects. Because of their toxicity, the development and isolation of natural antioxidants from plant species, especially edible plants, such as silymarin, polyphenols and flavonoids are in progress.

In vitro investigations have demonstrated that the antioxidant properties of flavonoids are linked with their capability for scavenging free radicals, chelating metals and inhibiting the activity of oxidases. Flavonoids are family of polyphenolic compounds found in fruits and vegetables. Flavonoids have wide biological properties including antibacterial, antiviral, anticancer, immune stimulant and antioxidant Effects. Flavonoids activity as antioxidants refers to their ability to transfer a hydrogen atom or an electron and to the possibility of their interactions with other antioxidants. Hesperidin is a flavonone glycoside, belonging to the flavonoid family. This natural product is found in citrus species. Hesperidin was reported to have many biological effects including antiinflammatory, antimicrobial, anticarcinogenic and antioxidant effects, and decreasing capillary fragility.

The flavonoid hesperidin may serve as a hydrogen donor for α-tocopherol radical, thus regenerating α-tocopherol, a key element of redox balance in biosystems. Hesperidin, in combination with a flavone called diosmin is used asDaflon®(Servier, France) to treat chronic venous insufficiency in Europe. Other biological effects of hesperidin are unknown.However, the structural activity relationship of hesperidin on their antioxidative activity has not been fully reported. In the present study, the structural activity effects of hesperidin on the antioxidative activities were investigated using a simple free radical scavenging system including, reducing power, Chelating activity on Fe2+, free radical scavenging, total antioxidant, superoxide radical scavenging, Hydrogen peroxide scavenging and hydroxyl radical scavenging activities in an attempt to understand its mechanism of action, which may pave the way for possible therapeutic applications.

Hesperidin has high antioxidant property. The antioxidant activity was evaluated using chemical and biological systems. The chemical assay evaluates the hesperidin capacity to sequester 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$). Biological Studies were done using the eukaryotic cells of superoxide-dismutase proficient and deficient Strains of *Saccharomyces cerevisiae* treated with hesperidin and the stressing agents hydrogen peroxide or paraquat (methylviologen; 1,1‘-dimethyl-4,4‘-bipyridinium dichloride). Hesperidin was able to reduce significantly the level of the free radical $DPPH^{\cdot}$ with similar efficacy of trolox (positive control). When the yeast cells were exposed to the flavonoid hesperidin before the stressing

agents, there was a significant increase in the survival of all strains. Paraquat induced higher catalase and superoxide dismutase than did hydrogen peroxide, which only increased catalase activity. Previous addition of hesperidin to these treatments was able to reduce significantly both enzymatic levels. These observations clearly demonstrate that hesperidin provides strong cellular antioxidant protection against the damaging effects induced by paraquat and peroxide hydrogen (*Patricia et al., 2005).*despite many reports on the medicinal or functional properties of hesperidin, substantial papers have not been published on the role of hesperidin in multiple organ failure/dysfunction. Therefore, in an effort to contribute further to the knowledge of hesperidin and on its traditional use, as a raw constituent of plants rich in hesperidin, and its sacred and rich history, the objective of the present study is designed to evaluate the possible role in protection against LPS induced neurodegeneration.

2. REVIEW OF LITERATURE

II.a. Lipopolysaccharide

Lipopolysaccharide (LPS), a major constituent of the outer membrane of gram-negative bacteria and is a key molecule in the pathogenesis of gram-negative endotoxemia, responsible for hemodynamic, hematological and metabolic changes, sepsis and septic shock (*Bone, 1991*). It is a large molecule, consisting of a polysaccharide attached to a lipid component, named lipid-A (Figure 2). The polysaccharide component consists of a core and a polymer of oligosaccharide molecules (O-antigens), which is immensely variable and specific to each bacterial strain. The polysaccharide core is bound to lipid-A through a specific sugar called 2-keto-3-deoxyoctulosonic acid (KDO) (*Rietschel, 1982*). Lipid-A is a peculiar bacterial lipid consisting of a phosphorylated β-1, 6-linked glucosamine disaccharide, to which long fatty acids are attached and is assumed to be responsible for the induction of expression of cytokines by LPS, although KDO and the polysaccharide component probably potentiate its activity (*Haeffner-Cavaillonet al., 1989; Rietschelet al., 1987*).

LPS function has been under experimental research for several years due to its role in activating many transcription factors, which become active after stimulation with LPS. LPS also produces many types of mediators involved in septic shock. It has become evident that there are diverse triggers like immunological insult, such as LPS; environmental toxins; endogenous disease proteins; neuronal injury; through which microglia are activated to exert their neurotoxicity. LPS-induced prototypical gram-negative endotoxemia is accompanied by contact system activation, complement activation, production of cytokines and other evidence of unregulated inflammatory responses (*Bone, 1996; Deitch, 1998*).

LPS induce inflammatory responses and parenchymal toxicity in liver diseases. LPS from *P. acnes causes* extensive liver injury mimicking fulminant hepatitis (*Ferluga& Allison, 1978*). Liver injury and massive hepatocellular damage can be also induced by a low dose of LPS in animals (*Hirokazu et al., 1999*).LPS exerts its effects by stimulating inflammatory cells and hepatic Kupffer cells to produce various pro-inflammatory cytokines, including tumour necrosis factor- alpha (TNF-a), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12) and interferon-gamma (IFN-g). It induces activation of caspases and apoptosis in hepatocytes prior to secondary necrosis and release of transaminases by activation of TNF receptor-1 (*Batey& Wang, 2000; Malhi& Gores, 2008*). The hepatic sinusoidal cells, particularly the fixed macrophages (Kupffer cells), are critical to normal endotoxin

detoxification. The initial damage in a number of injuries is to sinusoidal cells, which seriously impacts the ability of the liver to handle the ordinarily innocuous amounts of LPS coming from the gut. This marked increase in sensitivity to LPS, which may be of a magnitude of 10-fold to 1000-fold, leads to further hepatocytic damage and spill over of the endotoxins into the systemic circulation, resulting in the extrahepatic manifestations associated with liver injury (*James PN, 2010*).

The interaction between Kupffer cells, endotoxins, and hepatic injury remains a major area for productive investigation. It has long been known that liver endocytosis by Kupffer cells are a major phagocytic activity that removes many antigens from the portal and general circulation, including foreign particulate matter, immune complexes, and gut-derived endotoxin. There expected toxic hepatic and extrahepatic effects of endotoxin after liver injury (*James, 1975*).

LPS is known to induce SIRS condition in animal models and widely exploited for the search for mechanisms of SIRS-related conditions and exploration of drug development. LPS was shown to cause experimentally SIRS with acute renal failure. LPS induced a significant renal insufficiency as denoted by increase in plasma blood urea nitrogen (*Remick et al., 2000; Hollenberg et al., 2000*).

The administration of LPS to animals reproduces most of the clinical features of sepsis, including AKI, a condition associated with renal cellular dysfunction and apoptosis (*Stoyanoff et al., 2014*). Acute renal failure as a consequence of septicemia by LPS may be due to a decrease in glomerular blood pressure and renal blood flow, but it has been demonstrated in animal models that even in the absence of a reduction in blood pressure, LPS induces renal injury. There are studies to confirm that LPS cause marked renal damage, as demonstrated by an increase in plasma BUN. This was accompanied by a significant decrease in the vitamin E content of renal tissue and plasma (*Kadkhodaee&Qasemi, 2004*).

Administration of LPS to humans or laboratory animals induces a variety of physiological responses, neuroendocrine changes (*Bluthé et al., 1992; Dunn, 1992*) and modifications of behavior consisting of fever (*Kluger, 1991; Kozak et al., 1994*), anorexia and body weight loss, anhedonia, suppression of locomotor and exploratory activity and reduced social behavior (*Yirmiya et al., 1994; Yirmiya, 1996*) sleepiness, depression (sickness behavior) (*Bluthé et al., 1992; Hart, 1988*). Thus it has been suggested that immunological activation with LPS or cytokines themselves and may be interpreted by the CNS as a stressor, and that

the immune system may act as a sensory organ for non-cognitive stimuli such as bacteria, tumors, viruses etc, (*Anisman&Merali, 2002; Shen et al., 1999; Dunn & Vickers, 1994*).

It is well established that brain tissue is susceptible to degeneration in response to insult such as oxidative stress or infection. Peripheral administration of LPS initiates a robust inflammatory response, which is mediated by pro-inflammatory cytokine and free radical generation (*Feng et al., 1995*).

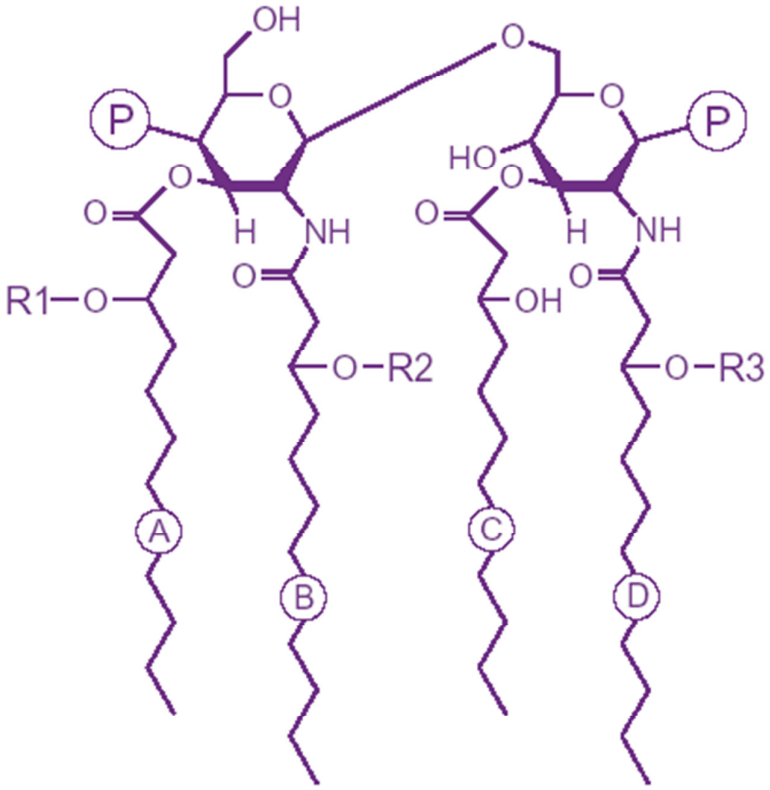
II.b. LPS: Oxidative Stress and Inflammation

Liver injury and dysfunction are typical features of the multiple organ failure complicating clinical gram-negative septic shock (*Ring &Stremmel, 2000*). The data presented here support a possible role of flagellin as an initiator of such abnormalities. Indeed, flagellin administration was associated with the occurrence of liver damage, indicated by an increase in plasma aminotransferases, significantly more pronounced than after LPS. While a possible mechanism underlying this effect was the marked oxidant stress generated by flagellin, additional studies, both morphological and functional, are required to more precisely define the mechanisms of flagellin toxicity on this organ (*Lucas et al., 2002*).

Within the liver, the LPS binds to LPS binding proteins which then facilitate its transfer to CD14 receptors on the surface of Kupffer cells, the resident macrophages of liver. Signalling of LPS through CD14 receptors is mediated by the downstream Toll-like receptor-4 and results in the production of two classes of potentially disastrous mediators: proinflammatory cytokines such as interleukins (IL-1, IL-6), tumour necrosis factor (TNF)-α and oxygen free radicals (*Luster et al., 1994*). Most of the toxicities of LPS, both in the liver and in the systemic circulation, have been related to the release of these toxic mediators (*Hartung&Wendel, 1991)*. LPS-induced increase in lipid peroxidation, which is an index of oxidative stress, has been described in several studies and is, reported to be both time- and dose-dependent. Lipid peroxidation was enhanced in rats as early as 45 min post-LPS infusion [100 mg/kg body weight (b.w.)] in many tissues including the liver, small intestine, stomach and abdominal aorta (*Yoshikawa et al*., 1994). Hepatic levels of malonaldehyde (MDA, a product of lipid peroxidation) increased fivefold within 16 h after LPS administration (15 mg/kg b.w.) and 3·4-fold 8 h after 30 mg/kg LPS administration (*Sugino et al., 1987*). Several studies have shown that LPS can cause liver glutathione (GSH) depletion in a dose-dependent manner (*Jaeschke et al., 1993*). This is thought to be secondary to enhanced efflux of GSH from the liver or acute depression of liver GSH synthesis. Also

oxidized glutathione (GSSG) is released from liver and other tissues during oxidative damage (*Kaur et al., 2006*).

Figure 1. Structure of Lipopolysaccharide from Different Bacterial Species



Species	A	B	C	D	R1	R2	R3
E.coli	14	14	14	14	14:0	12:0	H
Salmonella spp	14	14	14	14	14	12:0	14
*P.gigivatis**	15	17	16	17	H	16:0	H
N.Meningitidis	12	14	12	14	H	12:0	12:0
R.sphaoroidos	10	12	10	12	H	cis[45]12:1	H

*No phosphate e group on position 4' of the non-reducing glucosamine; ethanolamine at the other Phosphate group.

III. a. Plant Phenolics as Nutraceuticals

Flavonoids are a group of low molecular weight polyphenolic compounds of plant origin, many of which alter metabolic processes and have a positive impact on health (*Beecher, 2003*). They exhibit a variety of biological activities such as anti-inflammatory, antioxidant, antiviral and antitumor (*Middleton et al., 2000; Middleton & Kandaswami, 1992*).

Epidemiological studies have suggested positive associations between the consumption of phenolic-rich foods or beverages and the prevention of diseases (*Scalbert & Williamson, 2000*). These effects have been attributed to antioxidant components such as plant phenolics, flavonoids and phenyl propanoids among others (*Rice-Evans et al., 2004*).

III. b. Hesperidin

i. Sources

Hesperidin is a flavanone glycoside named after the term 'Hesperidium', referring to citrus fruits which are the main source of hesperidin. Hesperidin and its aglycone are common dietary flavonoids due to being large compounds of citrus fruits (alongside naringenin) and especially the peels and pericarp (*Katherine et al, 1993*) hesperidin has once been noted to comprise up to half of the flavonoid intake of Finland in surveys due to its prominence in the diet (at 28.3mg daily calculated as aglycones) (*Knekt et al., 2002*) and it could be argued that it is a Traditional Chinese Medicine (perhaps alongside naringenin) since the dried peels of citrus have been used medicinally and referred to as *Chimpi* (*Ito et al., 2013*).

A classical term 'Citrin' or 'Vitamin P' is used to refer to a mixture of Hesperidin and Eriodictoyl (another flavonoid), which were initially thought to have vitamin-like properties by having wound healing properties and treating scurvy; this was later attributed to Vitamin C *(Harold, 1940; Garg et al., 2001)*.

ii. Some other sources that are not in the citrus family

- *Boehmerianivea* (of the *Urticaceae* family) at 23.69 mg/100g (Sung et al., 2013). *Valerianawallichii* (of the family *Valerianaceae*) at 6.2+/-0.25mg/g (0.6%) of a water extract (*Katoch et al., 2012*).
- *Cyclopiasubternata* (Honey bush) at 0.504+/-0.495mg/g (range of 0.147–1.517mg/g) in the leaves and 1.559+/-0.289mg/g (range of 1.164–1.893mg/g) in the stems (*De Beers et al., 2012; Petrova et al., 2011*).
- *Codonopsispilosula* (*Qi et al., 2011*)
- *Schizonepetatenuifolia* (*Sohn et al., 2012*) at 12.0+/-0.4mg/g dried leaves (*Wang et al., 2012*).
- *Byrsonimacrassifolia* (of the family *malpighiaceae*) at 0.7mg/kg dry weight (*Herrera-Ruiz et al., 2011*).

Hesperidin, a flavanone-type flavonoid, is found abundant in the peel and membranous parts of citrus fruit (*Williamson, 1972*). Hesperidin is comprised of the flavanonehesperitin and the disaccharide rutinose and has been reported to have many biologically important properties,

including anti-inflammatory, antimicrobial, anticarcinogenic, antioxidant and capillary strengthening effects (*Garg et al., 2001*). Hesperidin has also been reported to possess anti hypercholesterolemic activity (*Son et al., 1991*), anti-inflammatory and analgesic activity (*Galati et al., 1994*), antifungal activity (*Krolicki et al., 1984*) and anticarcinogenic activity (*Yang et al., 1997*). The antioxidant activity and radical scavenging properties of hesperidin have been analyzed and reported by several investigators using a variety of assay systems (*Deng et al., 1997; Miller et al., 1997; Suarez et al., 1998, Malterud et al., 2000*). Further, hesperidin was found to be effective in protecting liposomes from UV-irradiation induced peroxidation, probably by scavenging the oxygen free radicals generated by UV-irradiation (*Bonina et al., 1996*).

Hesperidin, a flavanone glycoside copiously found in sweet orange and lemon, is an inexpensive by-product of citrus cultivation (*Garg et al., 2001*). Hesperidin is effectively used as a supplemental agent and helps to reduce edema or excess swelling in the legs due to fluid accumulation. It has been reported to possess significant anti-inflammatory, analgesic, antifungal, antiviral antioxidant, and anticancer activities (*Galati et al., 1994; Wacker&Eilmes, 1975*). A number of researchers have documented the antioxidant activity and radical scavenging properties of hesperidin (*Fraga et al., 1987; Jovanovic et al., 1994; Miller & Rice-Evans, 1997; Vaibhav& Kumar, 2010*).

Hesperidin was first isolated in 1828 by French chemist Lebreton from the white inner layer of citrus peels. Hesperidin is a compound in orange peels that gives the flavonoid hesperitin to the body, and this flavonoid mediates most benefits of hesperidin including an increase in circulation and possible brain protective effects. Hesperidin, alongside naringenin, is known as the main citrus flavonoids.

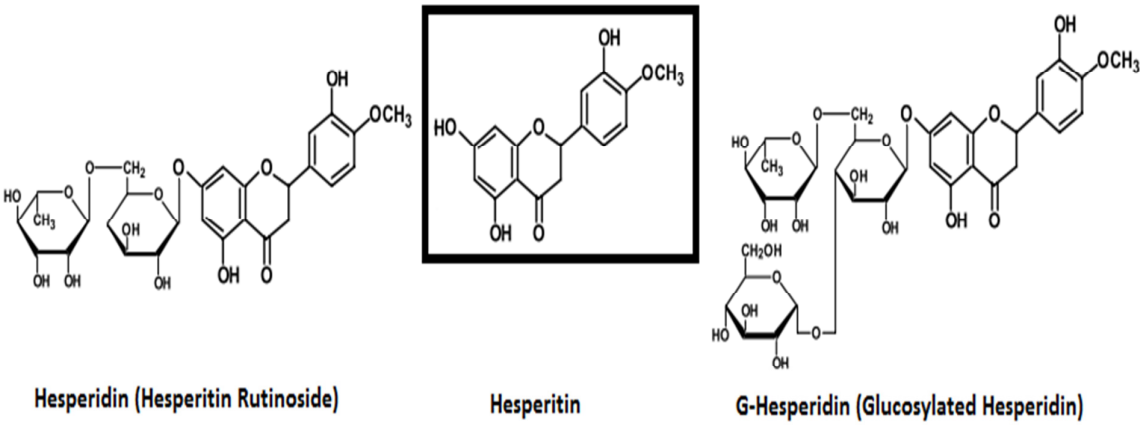
Hesperidin, a bioflavonoid, is an abundant and inexpensive by-product of Citrus cultivation. A deficiency of this substance in the diet has been linked with abnormal capillary leakiness as well as pain in the extremities causing aches, weakness and night leg cramps. No signs of toxicity have been observed with the normal intake of hesperidin or related compounds.

Both hesperidin and its aglyconehesperitin have been reported to possess a wide range of pharmacological properties. This paper reviews various aspects of hesperidin and its related compounds, including their occurrence, physical and chemical properties, analysis, pharmacokinetics, safety and toxicity and the marketed products available. A special

emphasis has been laid on the pharmacological properties and medicinal uses of these compounds. However, the mechanisms of action are still unclear (*Garg et al., 2001*)

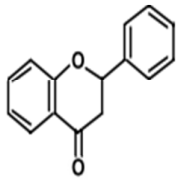
iii. Structure

As a glycoside (with sugars), it has an aglycone (without sugars) flavanone known as hesperitin (*5,7,3'-trihydroxy-4'-methoxyflavanone*) and can also be referred to chemically as *hesperitin-7-O-rutinoside* (as the sugar it is bound to is rutinose) or *hesperitin-7-O-rhamnosyl(1-6)glucoside* (since a 'rutinose' sugar is *6-O-α-L-rhamnosyl-D-glucose*, or simply a rhamnose sugar bound to a glucose).

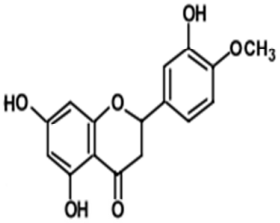


Hesperidin (Hesperitin Rutinoside) Hesperitin G-Hesperidin (Glucosylated Hesperidin)

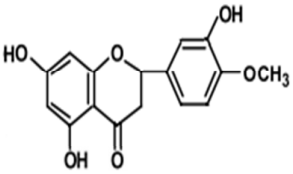
The main molecule here is hesperitin, but it is found in foods as hesperidin which pretty much acts like a hesperitinprodrug (gives the body hesperitin, but is better than hesperitin itself at doing so due to various reasons). G-Hesperidin is another synthetic prodrug for hesperitin. Hesperidin is known as a flavanone (a subset of flavonoid or bioflavonoid) due to there *not* being a double bond between the 2 and 3 carbon on the A ring, which is the rightmost vertical line one the middle hexagon (a double bond would mean that two vertical lines were there). This lack of a double bond means that the B ring (hexagon not immediately bonded to another, furthest right on the pictures below) is more perpendicular to the other two rather than adjacent like shown in the 'standard depiction'.



Flavanone Backbone



Standard Depiction of a Flavonoid



Accurate Depiction of a Flavanone Configuration

iv. Traditional Claims of Hesperidin

- Internal hemorrhoids, when used in combination with diosmin. The combination of hesperidin and diosmin seems to both significantly improve symptoms of hemorrhoids and also keep hemorrhoids, once healed, from coming back.
- Treating leg ulcers caused by poor circulation, when used in combination with diosmin. Hesperidin, in combination with diosmin and a compression dressing, seems to improve healing of small ulcers (less than 10 cm) caused by poor blood circulation.
- Reducing arm swelling in lymphedema following surgery for breast cancer.
- Varicose veins and improves circulation

v. Health Benefits of Hesperidin

- Hesperidin has antioxidant, anti-inflammatory, hypolipidemic, vasoprotective and anticarcinogenic and cholesterol lowering actions. Hesperidin can inhibit following enzymes: phospholipase A2, lipoxygenase, HMG-CoA reductase and cyclo-oxygenase.
- Hesperidin improves the health of capillaries by reducing the capillary permeability.
- Hesperidin is used to reduce hay fever and other allergic conditions by inhibiting the release of histamine from mast cells. The possible anti-cancer activity of hesperidin could be explained by the inhibition of polyamine synthesis.
- A study 'hesperidin, a citrus flavonoids, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice' by Hiroshige Chiba et al., 2003 showed that hesperidin added to the died not only lowered serum and hepatic cholesterol, but also inhibited bone loss by decreasing osteoclast number in ovariectomized mice. The molecular mechanism of the inhibitory effect of hesperidin on bone resorption is not clear.
-

III. c. Literature Review of Hesperidin

i. Antioxidant Properties of Hesperidin

Hesperidin possesses general protective effects against oxidative liver toxins, which are currently thought to be related to its antioxidant properties. Antioxidant enzymes such as SOD, catalase, and glutathione peroxidase seem to be fully preserved relative to control rats in groups administered with hesperidin (*Balakrishnan&Menon, 2007*). 50μM hesperitin was also neuroprotective against H_2O_2, but this was due to direct antioxidant properties and is not thought to be relevant to oral supplementation of hesperidin as it is too high a concentration (*Hwang & Yen, 2011*). There appear to be antioxidant effects in the brain where hesperidin reduces the increase in lipid peroxidation during cognitive damage, but this appears to be indirect through nitric oxide signaling (inhibition) rather than a direct antioxidant effect.Hesperidin at 100mg/kg orally to young and old rats for 90 days is able to partially reverse the worsening of antioxidant enzymes seen in heart tissue with aging, while the changes to these enzymes in young hearts were not significant (*Elavarasan et al., 2012*). Hesperidin was able to reduce significantly the level of the free radical DPPH· with similar efficacy of trolox (positive control) when tested with yeast cells that are exposed to paraquat (*Patricia et al., 2005*)

ii. Anti-inflammatory Properties of Hesperidin

Hesperidin is thought to have anti-inflammatory properties in fat cells, and while this technically does occur it happens at rather larger concentrations and has not yet been confirmed in a living model. Hesperidin significantly protects microglia activation and reduces the release of inflammatory cytokines proving the anti-inflammatory effect of hesperidin (*Kuppusamy et al., 2013*).

iii. Longevity

In a screening process in yeast for longevity promoting flavonoids, it was found that hesperidin was able to increase activity of the *sir2* gene (human homologue is SIRT1) and superoxide dismutase (SOD) resulting in less reactive oxygen species production; these benefits were not seen with the aglyconehesperitin (*Sun et al., 2012*).

Elsewhere, hesperidin (and rutin; the rhamnoside of Quercetin) have been noted to increase neural crest cell viability (neural progenitor cells) with peak efficacy at 20μM; the potency being comparable between both rhamnosides and not present with quercetin, implicating the rhamnoside group itself (*Nones et al., 2012*).

Hesperidin is an essential bioflavonoid and accessory nutrient to form a Vitamin C Complex with rutin (previously known as Vitamin P factor). They function synergistically with Vitamin C in regard to maintaining healthy capillaries, to help form collagen in connective tissue, to help heal wounds, and to support a healthy immune system. Rutin and/or hesperidin, when low, frequently result in predictable, and even side-specific medical problems that include a greater risk for vascular degeneration, bruising / capillary fragility, nose bleeds, varicose veins, periodontal bleeding, hemorrhoids and aneurism, with few individuals exhibiting optimal levels, despite mega-supplementation. One reason is the chemical interaction of other nutrients with flavonoids which can have a synergistic or inhibiting effect. Various drugs also interact with bioflavonoids and affect their efficacy.

iv. Neurology

Hesperidin was found not to possess any significant influence on dopaminergic, adrenergic, serotonergic transmissions in CNS. No known interactions with dopamine metabolism in the brain following hesperidin/hesperitin interventions.

v. Nitrergic Neurotransmission

50-100 mg/kg hesperidin orally for two weeks prior to an immobilization stress test was able to exert neuroprotective effects in a manner inhibited by Nitric Oxide donors (L-arginine) and potentiated by NOS inhibitors,suggesting negative regulation of nitric oxide signaling in the protective effects of hesperidin.

vi. Memory and Learning

In a model of memory dysfunction associated with ischemic stroke, supplementation of hesperidin (50-100 mg/kg oral for a week prior to ischemia) is able to minorly attenuate the memory loss seen in an elevated maze plus test in a manner that is associated with suppressing excessive nitric oxide signaling. The magnitude of memory loss (assuming that control was 100% reduction) was reduced to around a 20-60% reduction in a dose-dependent response, with neither dose normalizing to undamaged control *(Vaibhav& Kumar, 2010).*

vii. Depression

Injections of 0.1-1 mg/kg hesperidin (intraperitoneal) to mice subject to a force swim test is able to exert antidepressant effects while 10 µg/kg was ineffective on its own *(Filho et al., 2013)* and this has been replicated elsewhere in forced swim and tail suspension tests with a potency comparable to imipramine (15 mg/kg injection) and fluoxetine (32 mg/kg injection) at the dose of 0.3-1mg/kg *(Souza et al., 2013).*

viii. Anxiety and Stress

In a study which found antidepressant effects with intraperitoneal injections of hesperidin (0.1-1mg/kg), there was no apparent anxiolytic effect (*Filho et al., 2013*) and this lack of effect has been noted elsewhere with oral ingestion of 20-100 mg/kg hesperidin. Anxiolytic effects can be forced with high doses of hesperidin injections (2-30 mg/kg) in mice, thought to be related to the opioidergicsignalling (*Souza et al., 2013; Loscalzo et al., 2011*).

ix. Sedation and Sleep

Hesperidin has been described as a sedative flavanone*(Martinez et al., 2009)* possibly due to injections of hesperidin having suppressive effects in rodent locomotor tests *(Wasowski et al., 2012; Martinez et al., 2009; Loscalzo et al., 2011)* with an ED_{50} of 11.34+/-2.48 mg/kg *(Guzmán-Gutiérrez &Navarrete, 2009)* possible related to its opioidergicsignalling (via the μ-opioid receptor) *(Loscalzo, 2011)*. This does not occur with oral ingestion of hesperidin in the range of 20-100 mg/kg, possibly because orally ingested hesperidin is metabolized to hesperitin and the aglycone (hesperitin) does not possess this opioidergic activity (Wasowski et al., 2012). Injections may be synergistic with benzodiazepines ($GABA_A$ receptor agonists) as well, but this may not extend to oral intake *(Loscalzo et al., 2011).*

That being said, oral ingestion of hesperidin has been noted to signal in a manner dependent on κ-opioid receptors (*Filho et al., 2013*) and pharmaceutical κ-opioid agonists have been noted to have weak sedative effects. It is not known if oral supplementation of hesperidin confers sedative effects at higher doses (*Tsukahara-Ohsumi et al., 2011*).

x. Appetite and Food Intake

Ghrelin administration is known to enhance food intake *(Neary et al., 2004)* possible secondary to growth hormone secretion *(Laferrère et al., 2005)* and a Kampo formulation known as Rikkunshito is known to stimulate Ghrelin secretion *(Takeda et al., 2008; Matsumura et al., 2010)* via antagonism of the serotonergic 5-$HT_{2B/2C}$ receptors; in Rikkunshito, hesperidin is thought to be *active (Takeda et al., 2008).*

Hesperidin by itself at 5mg/kg orally to rats (where anorexia was induced by cisplatin) appears to attenuate the reduction in food intake seen with cisplatin by 59% while as a potency comparable to 1,000 mg/kg Rikkunshito but lesser than the synthetic 5-HT_{2C} receptor antagonist SB242084HCl (full negation), and the attenuation of anorexia seen with all of the three aforementioned was prevented with a GHS-R1a (Growth hormone secretagogue receptor) antagonist *(Yakabi et al., 2010)*, which is the receptor that Ghrelin acts upon (*Wellman et al., 2013*).

xi. Cardiovascular Health

Cardiac Tissue

Hesperidin at 100 mg/kg orally to young and old rats for 90 days is able to partially reverse the worsening of antioxidant enzymes seen in heart tissue with aging, while the changes to these enzymes in young hearts were not significant; a decrease in lipid peroxidation and protein carbonylation, and this was thought to be due to an increase in Nrf2 expression seen in the aged hearts that was decreased with aging (there was no increase in youth) (*Elavarasan et al., 2012*).

Blood Flow

In isolated endothelial cells, 1-10μM hesperidin has been noted to phosphorylated Ser1179 which resulted in its increased activity, *(Rizza et al., 2011)* which is thought to be due to phosphorylation from AMPK *(Chen et al., 1999)* or Akt *(Dimmeler et al., 1999)* on eNOS since both of those proteins were activated from hesperidin *(Rizza et al., 2011)*. Blocking either protein resulted in attenuation of eNOS activation from hesperidin and preventing ROS formation (with incubations of N-Acetylcysteine) blocked the effects, and similar to EGCG from Green Tea Catechins *(Kim et al., 2007)* it was attributed to thSrc protein known as Fyn (which acts on PI3K to then influence the two proteins, and is stimulated by the radical H_2O_2 (Griffin et al., 2000) where the hydroxyl groups have been noted to be critical to production of H_2O_2 from EGCG *(Auger et al., 2010)* and are thought to be the same for hesperidin *(Rizza et al., 2011)*.

Hesperidin has also been tested at a slightly higher concentration of 12.5μM in HUVEC cells, and the increase in nitric oxide production was significantly attenuated with estrogen blockers (*Liu et al., 2008*) hesperidin also increased eNOS transcription at 50μM, but these effects were not observed with naringenin (which was also estrogenic) while being observed with 10nM 17β-estradiol (*Liu et al., 2008*). Estrogen signalling via the alpha subset (ERα) (*Muller-Delp et al., 2003*) is also involved in producing nitric oxide (via increasing eNOS phosphorylation at Ser1179 (*Chambliss et al., 2000*) secondary to Src signalling, where Fyn is implicated by c-Src is more important (*Haynes et al., 2003*), which suggests that estrogenic signalling may underlie the observed benefits.

Hypolipidemic Activity

Hesperidin, the most important flavanone of Citrus sp., significantly increases HDL and lowerscholesterol, LDL, total lipid and triglyceride plasma levels in normolipidemic rats and in rats with diet- and triton-induced hyperlipidemia.

Cholesterol

G-hesperidin has been noted to suppress the secretion of Apolipoprotein B from HepG2 (liver) cells mildly at concentrations of 100µM and above when co-incubated; this may be due to donating hesperidin since it was active to the same degree at 25-50µM, and it appeared that the overall production of apolipoprotein B was reduced to a comparable level as the reduction in secretion *(Miwa et al., 2006)* This reduction of Apolipopotein B has been noted in vivo with 500mg of G-Hesperidin *(Miwa et al., 2005).*

Anti-Hypertensive

Hesperidin (LIES) is a flavonoid contained in citrus fruit peel. We investigated the effects of long-term administration of HES and its newly developed water soluble analogue, glucosyl hesperidin (GHES), to spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). Animals were fed with diets containing HES or GHES (30 mg/d/kg body weight) for 25 wk. While the daily food intake and the body weight of administered rats were not different from those of the non-administered control rats in both SHR and WKY through the experimental period, the blood pressure and heart rate of SHR administered HES or GHES for longer than 15 wk decreased as compared to the control group. The blood pressure and heart rate of WKY were not changed by the long-term administration of HES or CHES. These results suggest that HES and GHES have anti-hypertensive effects on hypertensive animals**.**

Anti-Coagulant

Two flavonoids, rutin and hesperidin, were investigated *in vitro* for anticoagulant activity through coagulation tests: activated partial thromboplastin time (aPTT), prothrombin time (PT) and thrombin time (TT). Only an ethanolic solution of rutin at the concentration of 830 µM prolonged aPTT, while TT and PT were unaffected. In order to evaluate whether the prolongation of a PTT was due to the decrease of coagulation factors, the experiment with deficient plasma was performed, showing the effects on factors VIII and IX. Since pharmacological activity of flavonoids is believed to increase when they are coordinated with metal ions, complexes of these flavonoids with Al(III) and Cu(II) ions were also tested. The results showed that complexes significantly prolonged aPTT and had no effects on PT and TT. Assay with deficient plasma (plasma having the investigated factor at less than 1%) revealed that complexes could bind to the coagulation factors, what may lead to a non-specific inhibition and aPTT prolongation. An effort was made to correlate stability of complexes with their anticoagulant properties.

Vasorelaxant Activity, Superoxide-Scavenging Ability and Cyclic Nucleotide Phosphodiesterase-Inhibitory Effects

This study investigated the vasorelaxant activity, superoxide radicals ($O_2^{\bullet-}$)-scavenging capacity and cyclic nucleotide phosphodiesterase (PDE)-inhibitory effects of hesperidin and hesperetin, two flavonoids mainly isolated from citrus fruits. Hesperetin concentration-dependently relaxed the isometric contractions induced by noradrenaline or by a high extracellular KCl concentration (60 mM) in intact rat isolated thoracic aorta rings. However, hesperetin did not affect the contractile response induced by okadaic acid. Hesperetin did not scavenge $O_2^{\bullet-}$ generated by the phenazinemethosulfate (PMS)-reduced β-nicotinamide adenine dinucleotide (NADH) system. Hesperetin significantly reversed the inhibitory effects ofNA and high KCl on cyclic nucleotide (cAMP and cGMP) production in cultured rat aortic myocytes. Hesperetin preferentially inhibited calmodulin (CaM)-activated PDE1 and PDE4 isolated from bovine aorta with IC_{50} values of about 74 μM and 70 μM respectively. In contrast, the 7-rhamnoglucoside of hesperetin, hesperidin, was inactive in practically all experiments, although it inhibited basal and cGMP-activated PDE2 isolated from platelets (IC_{50} values of 32±4 μM and 137±34 μM respectively). These results suggest that the vasorelaxant effects of hesperetin are basically due to the inhibition of PDE1 and PDE4 activities.

x. Bone

The purpose of this study was to examine whether hesperidin inhibits bone loss in ovariectomized mice (OVX), an animal model of postmenopausal osteoporosis. Forty 8-wk-old female ddY mice were assigned to five groups: a sham-operated group fed the control diet (AIN-93G), an OVX group fed the control diet, an OVX+HesA group fed the control diet containing 0.5 g/100 g hesperidin, and an OVX+HesB group fed the control diet containing 0.7 g/100 g α-glucosylhesperidin and an OVX+17β-estradiol (E_2) group fed the control diet and administered 0.03 μg E_2/d with a mini-osmotic pump. After 4 wk, the mice were killed and blood, femoral, uterine and liver were sampled immediately. Hesperidin administration did not affect the uterine weight. In OVX mice, the bone mineral density of the femur was lower than in the sham group ($P< 0.05$) and this bone loss was significantly prevented by dietary hesperidin or α-glucosylhesperidin. The Ca, P and Zn concentrations in the femur were significantly higher in the hesperidin-fed and E_2 groups than in the OVX group. Histomorphometric analyses showed that the trabecular bone volume and trabecular thickness in the femoral distal metaphysis were markedly decreased ($P< 0.05$) by OVX, and

α-glucosylhesperidin significantly prevented this bone loss. Furthermore, hesperidin decreased the osteoclast number of the femoral metaphysis in OVX mice, as did E_2. Serum and hepatic lipids were lower in mice that consumed the hesperidin-containing diets ($P<$ 0.05) than in the OVX group fed the control diet. These results suggest a possible role for citrus flavonoids in the prevention of lifestyle-related diseases because of their beneficial effects on bone and lipids.

xi. Radioprotective Effect

The present study was aimed to evaluate the radioprotective efficacy of hesperidin, a flavonone glycoside against X-ray radiation-induced cellular damage in the liver of Swiss albino mice. The first phase of the study was carried out to fix the effective concentration of hesperidin by performing a 30 days of survival studies using different graded doses [12.5, 25, 50 and 100 mg/kg body weight] of hesperidin administered orally to mice via intragastric intubations for seven consecutive days prior to exposure of whole body radiation (10 Gy). Based on the results of survival studies, the effective dose of hesperidin was fixed which was then administered to animals orally via intragastric intubations for seven consecutive days prior to exposure of whole body radiation (4 Gy) to evaluate its radioprotective efficacy by performing various biochemical estimations, comet assay, DNA fragmentation assay and histopathological studies in the liver of Swiss albino mice. The results indicated that radiation-induced decrease in the levels of endogenous antioxidant enzymes and increase in lipid peroxidative index, DNA damage and comet parameters were altered by pre-administration with the effective dose of hesperidin [25 mg/kg body weight] which restored the antioxidant status to near normal and decreased the levels of lipid peroxidative index, DNA damage and comet parameters. These results were further confirmed by histopathological examinations which indicated that pre-administration with the effective dose of hesperidin reduced the hepatic damage induced by radiation. Thus the current study shows hesperidin to be an effective radioprotector against radiation induced damage in the liver of mice.

xii. Anti-Ulcer

Hesperidin and neohesperidindihydrochalcone show a marked capacity to reduce the ulcer index in cold-restraint induced ulcer in a clear dose-related manner. The amount of gastric mucus and total protein were not modified, but there was a significant increase in hexosamine content. When the acute ulcer was induced by absolute ethanol HESP was inactive. These results suggest that mucus is not involved in the antiulcer activity. In both experimental

models, HESP and NEOHESP did not produce an increase in total PGE_2, suggesting that this mechanism of action is not involved in the antiulcer activity of these compounds.

xiii. Peripheral Organ Systems

Liver

Following irradiation, 50-100 mg/kg oral ingestion of hesperidin for one week after irradiation therapy has been noted to reduce the elevation of liver enzymes and reduce lipid peroxidation to near normal levels *(Pradeep et al., 2008)* which has been noted elsewhere *(Pradeep et al., 2012)*. In rats administered the liver toxin TCDD, 50 mg/kg hesperidin daily alongside the TCDD, was able to attenuate the oxidative and inflammatory changes which were thought to underlie the protective effects on the organ *(Bentli et al., 2013)*. Oral ingestion of hesperidin (25mg/kg) daily for 22 weeks in rats prevented an increase in liver enzymes (84-89% normalization on ALT, AST, and ALP) and also significantly attenuated the rise in other biomarkers of toxicity (tissue phospholipids, free fatty acids, and cholesterol) seen in the liver, lung, and kidneys of these rats *(Balakrishnan&Menon, 2007)*.

Lungs

Hesperidin is thought to be beneficial for the lungs due to men with higher hesperidin intakes having lower mortality from cerebrovascular disease and lung cancer with lower incidences of asthma *(Knekt et al., 2002)* and that hesperitin possesses a selective PDE4 inhibitory effective *in vitro (Ko et al., 2004)*.

Hesperidin (30-100 μM/kg oral ingestion) two hours before and twice after an after way hyperresponsiveness test in mice (for airway allergies) noted a mild attenuation of symptoms suggesting antiallergic effects, and this was associated with less infiltration of immune cells (leukocytes and macrophages) into lung tissue; *(Yang et al., 2012)* and this has been replicated elsewhere with 10-30 mg/kg (but not 5mg/kg) hesperidin an hour before stimulation *(Wei et al., 2012)* Since a variant (HDME) that was a more effective PDE inhibitor was more effective, implicating that as a mechanism of action *(Yang et al., 2012)*.

Kidneys

In rats given 100-200mg/kg hesperidin daily for a week, there are protective effects against subsequent injections of cisplatin with more efficacy in reducing lipid peroxidation (TBARS; a 95% attenuation at 100mg/kg and full normalization with a further 18% reduction at 200mg/kg) and protein carbonyls (79-84% normalization) rather than normalizing creatinine (30-53%) and BUN (34-57%); these changes were associated with full preservation of SOD

and glutathione-S-transferase activity at both doses. In control rats, 100-200mg/kg hesperidin trends to reduce oxidation but it do not reach significant levels *(Sahu et al., 2013)*.

Testes

In doxorubicin induced testicular toxicity, hesperidin (25-100mg/kg) daily give times weekly for five weeks is able to attenuate oxidative changes induced by doxorubicin and alongside that also normalized signs of toxicity such as abnormal sperm cells and testicular histopathology *(Trivedi et al., 2011)* In a mouse model of androgen deficiency, supplementation of either hesperidin (0.5% of the diet) or G-hesperidin (0.7%) failed to reduce the atrophy of the testicles that occurred from the androgen deficiency *(Chiba et al., 2014)*.

3. SCOPE OF THE WORK

The present study was aimed to evaluate the protective effect of hesperidin against LPS-induced Neuroinflammation and forced-swimming-stress-induced alterations in biochemical, behavioral, and histopathological changes in rats.

Stress is a very crucial factor in the maintenance of health and disease (*McIlroy & Craig., 2004*). Stress induces changes in emotionalbehavior and anxiety like state, which are associated withoxidative damage, that is, free radical damage (*Fontella et al.,2005 ; Firuzi et al., 2006*). Acutestress triggers numerous cellular cascadesthat lead to increase in ROS production (*liu et al., 1996*). Because of thebrain high oxygen consumption, abundant lipid content,and relative paucity of antioxidant enzymes, the centralnervous system is highly vulnerable to free radical damage *(Halliwell & cutteridge., 1985*). Immobilization stress has also been reported to induce2-3-fold higher rise of plasma cortisol level; increasedcortisol level has been linked with anxiety-like behavior (*Bristowet al.,2007; Goyal et al., 2007*). It is been reported that stress triggers the motoralteration in different animal models and central nucleus ofamygdala is important in modulating affective response tostress (*LeDoux ., 1998*).

Natural products such as bioflavonoids possess very goodantioxidant property and inhibit lipid peroxidation in biological membranes (*Maridonneau-Parini et al., 1986*). Hesperidin is a such natural bioflavonoidthat possesses very good antioxidant property (*Haenen et el., 1997*)and it has been proved to be very effective in various neurobehavioral diseases .Stress has been known to cause serious disturbances in behavior and cognition.

With this background, the present study was designed toinvestigate the possible neuroprotective effect of hesperidin against acute immobilization-stress-induced anxiety-like behavior and associated oxidative damage in mice.

Neuroinflammation induced by LPS plays a major role and has a cascading effect on the functioning of the brain. There are, indeed, a multitude of paradigms assessing various aspects of the neurodegeneration like behavioral assessment, biochemical analysis and histopathological evaluation. Till now, some of the paradigms have not been used at all in the evaluation of hesperidin against neurodegeneration of adult rats in endotoxemia.

In this study, an attempt has been made to investigate whether pretreatment of animals with hesperidin for 30 days and then a single exposure to LPS-induced endotoxemia and immobilization stress would protect the animals from the corresponding effect on functions

of brain, using a series of behavioral tests, *in vivo* brain antioxidant defense elements and histological studies.

Animals were subjected to endotoxemic (stress) and an acute stress (forced swimming cold stress, a model for physical stress). We anticipate that the battery of tests used in the presence study, could contribute to the evaluation of hesperidin against neurodegeneration induced endotoxemia and acute stress and may shed an insight into the mechanism of action. Hence, a spatial attention is focused to understand the treatment of neurodegeneration by natural flavonoids, preferably hesperidin.

Therefore, to evaluate the traditional and modern claims of hesperidin, we have attempted to investigate the effect of LPS-induced endotoxemic stress+ acute stress on several aspects of brain metabolism including the measurement of brain malondialdehyde (MDA), reduced glutathione (GSH) contents, catalase (CAT) and superoxide dismutase (SOD) activities, as indices of oxidative stress; brain cholesterol and phospholipids that reflect membrane integrity and synthetic capacity.

In addition to these, behavioral studies such as locomotor activity, elevated plus maze, open-field test and forced swim test andhistopathological analysis of rat brain were studied.

4. Plan of Work

The present study is designed to assess the protective effect of marketed product of hesperidin in LPS- and stress-induced neurodegenerative rat model. The work has been planned to be carried out in the following phases as outlined below:

Phase: 1

I.a. Procurement of hesperidin

Hesperidin will be procured from sigma Aldrich ltd., Bangalore, India.

Phase: 2

II.a. Pharmacological studies

b. Animals

Healthy inbred male Sprague-Dawley rats weighing between 125-150g received from the central animal house of the institute and will be used for the study. The institutional animal ethical committee under the regulation of CPCSEA guidelines, New Delhi, has approved the protocol for the animal study.

c. Induction of stress

i. Endotoxemic stress:

LPS will be used to induce endotoxemia-induced neurodegenerative / oxidative stress in rats and the effect of Hesperidin on the behavioral and biochemical parameters along with histopathological analysis will be studied.

ii. Cold Physical stress:

Forced swimming induced stress model will be employed to induce immobilization stress induced anxiety in rats.

d. Experimental Protocol:

The rats were divided into nine groups of 6 animals each. The treatment period comprised of 30 days in four groups, and stress period for chronic unpredictable stress (CUS) was 10 days and for acute stress was 2 h. They were grouped under the following regimen:

Group-I, served as control and it received 0.9% normal saline.
Group-II, received LPS + acute stress.
Group-III, received diazepam (2mg/kg) + LPS + acute stress.
Group-IV, pretreated with Hesperidin (50mg/kg) + LPS + acute stress.
Group-V, pretreated with Hesperidin (100mg/kg) + LPS + acute stress.
Group-VI, pretreated with Hesperidin (200mg/kg) + LPS + acute stress.

Drug treatment

Groups	Drugs	Drug treatment	Routes	Dose
1	Control(saline)	30 days	p.o.,	-
2	LPS + acute stress	30 days	i.p., + cold stress	1.5 mg/kg
3	Diazepam + LPS + acute stress	30 days	p.o.,	2mg/kg
4	Hesperidin-I	30 days	p.o.,	50mg/kg
5	Hesperidin-II	30 days	p.o.,	100mg/kg
6	Hesperidin-III	30 days	p.o.,	200mg/kg

d. Evaluation of general behavioral alterations in Stress induced rats:

i. General behavioral parameters

- Body weight
- Food intake
- Water intake

ii. Behavioral studies

i) Test for anxiety studies

- Ambulatory behavior test
- Elevated plus maze
- Open field test

Phase: 3

III.a. Biochemical Analysis

i. Specimen Preparation

At the end of the behavioral studies, the animals were anesthetized with either and sacrificed by cervical dislocation, the brains quickly removed, weighed, rinsed in ice-cold isotonic saline and one half of the brain was processed as follows:

One half of the brain tissue homogenate was prepared with ice cold Tris-KCl buffer (0.05M Tris and 1.15% KCl, pH 7.4), using Elvehjem hand homogenizer, fitted with Teflon pestle. The homogenate was centrifuged at 2000 rpm at 4°C for 15 minutes, and the supernatant was used for the biochemical analysis.

The other half of the brain was stored in FAM (40% formaldehyde, acetic acid and methanol in the ration of 1:1:8), and used for histopathological analysis.

III. b. *In-vivo* Studies

Enzymatic and non-enzymatic antioxidants play a crucial role in neurodegeneration. Hence, the effects of hesperidin and the following antioxidant defense elements in brain were also be measured:

- **i.** Superoxide dismutase (SOD)
- **ii.** Catalase (CAT)
- **iii.** Total glutathione (GSH)
- **iv.** Malondialdehyde (MDA)
- **v.** Brain phospholipids (BPL)
- **vi.** Brain cholesterol (BCh)

Phase: 4
IV.a. Histopathological Examinations

The other half of the brain will be extracted and embedded in paraffin and saggital section of 5 µm thickness of brain and the extent of anatomical damage in the appropriate was analyzed using hematoxylin/eosin. The stained sections of brain tissues will then assessed for the extent of protective actions of drugs.

Phase: 5
5.a. Statistical Analysis

The data will be expressed as mean ± SEM from six observations in each group. The values were analyzed by one-way analysis of variance (ANOVA), followed by Dunnet's multiple comparison posttests.

5. Materials and Methods

4.a. Chemicals Used

Ferrous chloride, ferric chloride, thiobarbituric acid (TBA), calf thymus DNA, trichloroacetic acid (TCA), phenazinemethosulphate, potassium ferricyanide, were purchased from Sigma Aldrich Co., St Louis, USA. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sisco Research Laboratories Pvt Ltd., Mumbai, India. Ascorbic acid, nitrobluetetrazolium (NBT) and butylatedhydroxyanisole (BHA), methanol, ammonium acetate and formic acid (AR grade) were obtained from S.D. Fine Chem Ltd., Biosar, India. Ammonium thiocyanate was purchased from E Merck Ltd., Mumbai, India. Lyophilized lipopolysaccharide powder from *Escherichia Coli*, serotype 055:B5 (containing ≥ 10,000 endotoxin units / mg LPS) and potassium dihydrogenortho phosphate (AR grade) were purchased from Sigma Chemical Company, New Delhi, India.

4.b. Induction of LPS endotoxemia

Endotoxemia-mediated neurodegeneration can be elicited by systemic injection of LPS, triggering neuronal death with increased release of glutamate and NO production. Furthermore endotoxemic shock involves increase in free radicals, lipid peroxidation, decreased mitochondrial function and cytokine release. Endotoxin and cytokines have been reported to drive a cascade of mediators, which result in degeneration. Number of therapeutic agents modifying the above mentioned parameters may be tried in order to improve the outcome of endotoxemic injury.

The dose of LPS required to produce optimal endotoxemia, with relevance to our study, is standardized by testing different doses of LPS (10, 5, 2.5 mg/kg) in rats until a desired endotoxemic response was observed. LPS toxicity was induced in non-fasted male SD rats, weighing between 125-150 g, by single intraperitoneal injection of 1.5mg/kg of LPS in saline 1 hour after LPS administration. Hesperidin was administered at the dose levels of 50, 100 and 200 mg/kg body weight, up to 30 days and the effect was compared with diazepam (2mg/kg).

4.c. Drug Treatment

The following drugs were administered to the rats as per the schedule given below:

Groups	Drugs	Drug Treatment	Routes	Dose
1.	Control	30 days	p.o.,	-
2.	Diazepam	30 days	p.o.,	2mg/kg
3.	LPS + acute stress	31st day	i.p.,	1.5 mg/kg
4.	Hesperidin-I	30 days	p.o.,	50 mg/kg
5.	Hesperidin-II	30 days	p.o.,	100 mg/kg
6.	Hesperidin-III	30 days	p.o.,	200 mg/kg

The animals were pretreated with hesperidin and standard drugs for 30 days. On the last day (31stday), the animals were administered with an intraperitoneal injection of LPS, and thereafter the animals were subjected to the experimental protocol, as mentioned in the plan of work.

4.d. Evaluation of behavioral alteration in LPS-treated rats

The animals in this model were subjected to the same behavioral procedure immediately following treatment with hesperidin and standard drugs, 1 hour after intraperitoneal administration of LPS. The rats were observed for the behavioral changes during the entire treatment period.

4.e. Pharmacological Evaluation

4.e.1.General behavior

The psychological andphysiological effects of immune activation following LPS injection resemble the characteristics of depression. The essential features of depression are depressed mood and loss of interest or pleasure in all, or almost all activities (anhedonia).

i. Evaluation of general behavioral alterations in Stress induced rats:

The animals were subjected to the following behavioral procedures initially and after induction of stressors. During the entire period of study the animals were observed for any changes in behavior and suitably noted.

4.e.2.Test for Anxiety Studies

i.Actophotometer

The actimeter test was performed independently as a test to record the effects of the drugs on the spontaneous locomotor activity of rats using a photo-electric actimeter, 1h after administration of drug.The apparatus consist of stainless steel box containing transparent cages (270x20x110cm) in which the animal's horizontal activity in

measured by two light beams connected to a photoelectric cell. The total number of beam crossings will be recorded over a period of 5 min (*Ramanathan et al., 2007*).

ii. Elevated Plus Maze

The elevated plus maze (EPM) was performed on rats as a standard test of fear and anxiety, where anxiety-related behavior is measured by the degree to which the rodent avoids elevated, enclosed arms of the maze and exhibits defense behaviors such as head dips and scanning posture. The maze was elevated 50 cm above ground and consisted of four arms 48cm in length. Two opposing arms were open with no walls, while 48cm high walls enclosed the other two opposing arms. There was a 10x10cm open area at the confluence of the four arms. Testing procedures followed the behavioral neuroscience protocol for EPM (*Current Protocols in Neuroscience 2001, John Wiley and Sons: Supplement 10 section 8.3.6.Basic Protocol 4*).

One hour before animals were put in the maze, they were placed in a darkened holding room followed by testing in a dimly lit room. Animals were placed individually in the center facing an open arm, and the rodent's behavior recorded by video camera. Tapes were scored by a researcher blind to treatment for (1) first arm preference, (2) entries into closed arms and (3) entries into open arms.

iii. Open field test

An open field apparatus similar to that of *Bronstein (1972)*, made of plywood and consists of a square (61×61×61cm) was used. The entire apparatus is painted black except for 6mm white lines that divided the floor into 16 squares. The open field was lighted by a 100W bulb focusing onto the field from a height of about 100cm from the floor. The entire room was kept dark during the experiment. Each animal was centrally placed in the test apparatus for 5 min and the behavioral aspects of anxiety such as ambulation, rearing, self-grooming, defecation and activity in central squares were recorded. The open-field apparatus was then cleaned using 5% ethanol before introducing the next animal, to preclude the possible cueing effects of odors left by precious subjects. To minimize the possible influences of circadian changes on rat open-field behavior, control and experimental animals were intermixed.

Forced swimming induced stress:

In order to produce swimming induced stress, rats were made to swim in a cylinder (30cm diameter and filled to a height of 20 cm with 15 cm of space above the head of the rat) for 30 min. session in a day for ten consecutive days *(Ferry et al., 1991).*

EXPERIMENTAL PROTOCOL

The rats were divided into nine groups of 6 animals each. The treatment period comprised of 30 days in four groups, and stress period for chronic unpredictable stress (CUS) was 10 days and for acute stress was 2 h. They were grouped under the following regimen:

Group-I, served as control and it received 0.9% normal saline.
Group-II, received LPS + acute stress.
Group-III, received diazepam (2mg/kg) + LPS + acute stress.
Group-IV, pretreated with Hesperidin (50mg/kg) + LPS + acute stress.
Group-V, pretreated with Hesperidin (100mg/kg) + LPS + acute stress.
Group-VI, pretreated with Hesperidin (200mg/kg) + LPS + acute stress.

4.6 Biochemical Analysis

4.6.1 Specimen Preparation

At the end of the behavioral studies, the animals were anesthetized with either and sacrificed by cervical dislocation, the brains quickly removed, weighed, rinsed in ice-cold isotonic saline and one half of the brain was processed as follows:

One half of the brain tissue homogenate was prepared with ice cold Tris-KCl buffer (0.05M Tris and 1.15% KCl, pH 7.4), using Elvehjem hand homogenizer, fitted with Teflon pestle. The homogenate was centrifuged at 2000 rpm at 4°C for 15 minutes, and the supernatant was used for the biochemical analysis.

The other half of the brain was stored in FAM (40% formaldehyde, acetic acid and methanol in the ration of 1:1:8), and used for histopathological analysis.

4.6.2 *In-vivo* Studies

a. Superoxide dismutase (SOD)

The activity of SOD was estimated following the method of (*Kakker et al., (1984).* The measurement of SODinvolves the inhibition of the formation of the blue colored formozan dye from nitro blue tetrazolium (NBT), in the presence of phenazinemethosulphate (PMS) and reduced nicotinamide adenine dinucleotide (NADH). The incubation mixture consisted of sodium pyrophosphate buffer (pH 8.3; 0.052 M; 1.2ml), PMS (186 µM), NBT (300 µM) and NADH (780 µM; 0.2ml). The reaction was initiated by the addition of NADH; followed by incubation for 90 sec at

37°C. The reaction was terminated by the addition of glacial acetic acid (1ml), n-butanol (4ml), shaken vigorously, centrifuged at 4000rpm for 1min and the upper butanol layer was read at 560nm, against butanol blank.

b. Catalase (CAT)

The activity of CAT was measured according to the method of *Beers &Sizer (1952).* CAT measurement was done based on the ability of CAT to inhibit oxidation of hydrogen peroxide (H_2O_2). 2.25ml of potassium phosphate buffer (65 mM, pH 7.8) and 100µl of the brain homogenate or sucrose (0.32 M) were incubated at 25°C for 30 minutes. H_2O_2 (7.5 mM; 650µl) was added to initiate the reaction. The change in absorption at 240 nm was measured for 2-3min. dy/dx for every minute for each assay was calculated and the results are expressed as CAT units of protein.

CAT (U) in 100µl of sample = dy/dx **X** 0.0003 **/** 38.3956 **X** 10^{-6}

c. Total glutathione (GSH)

The following working solutions was prepared from the stock buffer for the estimation of GSH as follows: (1) 125mM sodium phosphate, 6.3mM sodium EDTA, adjusted to pH 7.5; (2) 0.3mM NADPH, 6mMdinitrothiobisnitroso benzoic acid (DTNB) and (3) approximately 50 units of glutathione reductase per ml and stored at 4°C. During the assay, 700µl of NADPH, 100µl of DTNB, 25µl of glutathione sample, 10µl of glutathione reductase were incubated at 30°C and 165µl of deionized water were incubated at 30°C and the absorbance was read immediately at 420nm (*Anderson, 1985).*

d. Lipid peroxide (TBARS)

*Ohkawa et al., (1979)*methodwas used to estimate the total amount of lipid peroxidation (LPO) product. LPO was estimated in terms of TBARS and malondialdehyde (MDA) was taken to represent the TBARS. The incubation mixture consisting of 0.5ml of supernatant brain homogenate, 0.2ml of 8% sodium dodecyl sulphate, 1.5ml of 20% acetic acid solution (adjusted to pH 3.5 with 1NaOH / 0.1N HCl) and 1.5ml of 0.9%.

Aqueous solution of thiobarbituric acid (adjusted to pH 7.4 with 1N NaOH / 0.1N HCl) was made up to 5.0ml with double distilled water and then heated in boiling water bath for 30 minutes. After cooling, the redchromogen was extracted into 5ml of

the mixture of n-butanol and pyridine (15.1v/v) centrifuged at 4000 rpm for 10 minutes. The absorbance of organic layer was measured at 532 nm. 1, 2, 3, 3-tetraethoxypropane (TEP) was used as an external standard and the levels of lipid peroxide was expressed as µmoles of MDA / g protein. The calibration curve of TEP was prepared by the above procedure taking 80-240nmoles of TEP as standard over which, linearity was obtained.

e.Brain phospholipids (BP)

Brain lipids were extracted (*Bligh & Dyer, 1959*) and used for estimation of cholesterol (kit by bio Merieux, France) (*Allain et al., 1974*), and phospholipids (*Raheja et al., 1973*; *Vaskovsky&Kostetsky, 1968*).

f. Estimation of Lipids

For quantitative lipid extraction the lipid withheld in the tissue residue is recovered by blending the residue and filter paper with 100 ml chloroform. The mixture is filtered through the original Buchner funnel and the blender jar and residue are rinsed with a total of 50 ml chloroform. This filtrate is mixed with the original filtrate prior to removal of the alcoholic layer. A portion of the lipid extract containing 100-200 mg lipid is evaporated to dryness in a tared flask and the weight of the lipid residue determined. Evaporation, facilitated by a stream of nitrogen, is carried out in a water bath at 40-50" C and the residue is dried over phosphoric anhydride in a vacuum desiccator. After weighing, a small volume of chloroforrn is added to each flask to detect the presence of non-lipid material (insoluble). If non-lipids are present, the chloroform is carefully decanted and the flask rinsed three times with chloroform. The dry weight of the residue is determined and subtracted from the initial weight. The lipid content of the sample is calculated as follows (*Bligh & Dyer, 1959*):

Total lipid = weight of lipid in aliquot X volume of chloroform layer / volume of aliquot**.**

g.Estimation of Brain Cholesterol

Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase (EC 3.1.1.13). The free cholesterol produced is oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of hydrogen peroxide, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm. Optimization studies were

performed for each of the components of the cholesterol assay. A native human serum pool with a total cholesterol content of 500 mg/dl was used in the optimization of cholesterol ester hydrolase, sodium cholate, and pH; a 600 mg/dl cholesterol standard in isopropanol was used in the remaining optimization studies. When we had ascertained the optimum concentration of a particular ingredient it was maintained at that concentration while the next ingredient was optimized. In the manual assay of total cholesterol by the present method, 30 µL of serum is incubated with 3.0ml of reagent for 10 mm at 37 °C and the absorbance at 500 nm is measured vs. a reagent blank. Concentrations of unknown samples are determined from a standard curve constructed by using cholesterol standards in isopropanol *(Allain et al., 1974).*

h. Estimation of Phospholipids

The phospholipids are estimated using a modification of the spray reagent described by *Vaskovsky & Kostetsky (1968)* after separation by thin-layer chromatography and elution from the silica gel, are heated with a chromogenic solution that is a modification of a spray reagent formulated by *Vaskovsky & Kostetsky(1968)*. The absorbance of the colored complex was read at 710 nm, and it followed Beer's law in the range of 1-10 microgram of phospholipid phosphorus *(Raheja et al., 1973).*

4.8 Histopathological Examinations

At the end of the behavioral procedures, the rats were scarified as described above. The brain of all the animals are extracted and embedded in paraffin. A sagital section of 5µm thickness of was carefully prepared using microtome and the tissues were stained using hematoxylin/eosin. The stained sections of brain tissues were then assessed for the extent of protective actions of drugs.

4.9 Statistical Analysis

The data are expressed as mean ± SEM from six observations in each group. The general behavioral data was subjected to one-way analysis of variance (ANOVA), followed by Dunnet's multiple comparison posttests. A probability level (p) of value of less than 0.05 was considered to be statistically significant. The statistical analysis was carried out using GraphPad Prism version 4.03 for Windows (GraphPad Prism Software, San Diego, California, USA).

6. RESULTS

5.1. Effect of Hesperidin and Diazepam on behavior Studies in LPS-Treated Rats

5.1.1. General Behavior

After induction of endotoxemia, the animals were observed for general behavior up to 1 hour. Endotoxemia may result in psychological and physiological changes in behavior, attributable to neurodegeneration. The animals were observed for a period of 45 minutes following administration of LPS after pretreated with hesperidin and diazepam for the changes in general behavior. Rats treated with LPS exhibited a prolonged suppressive behavior in comparison to the control rats. Pretreatment with hesperidin for 30 days, resulted in profound calmness.

5.2. Anti-anxiety Studies

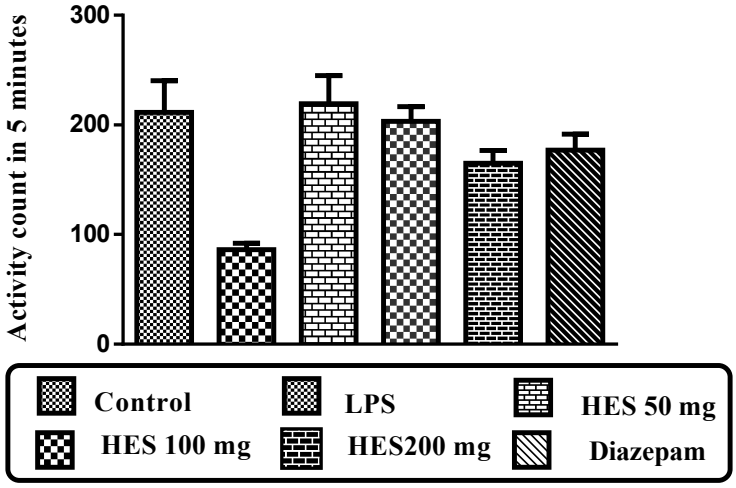
a. Effect of Hesperidin and Diazepam on Locomotor Activity in LPS-Treated Rats Using Actophotometer

Locomotor activity of LPS-treated animals was determined with an actophotometer and the results are shown in figure 1 and table 1. When tested on an actophotometer, a significant decrease in movement levels was seen in LPS-treated rats (86.33±6.15), when compared with control animals (211±29.15). A significant improvement in movement activity was seen in LPS-treated animals administered with hesperidin at different doses (50 mg/kg: 219.3±25.57; 100 mg/kg: 203±13.5; 200 mg/kg:165.1±11.37).The locomotor activity of rats treated with Diazepam (177±14.66) was found to be significantly better.

Table1. Effect of Hesperidin and Diazepam on LPS Treated Rats by Locomotor Activity

Groups	Number of Movement (in 5 min)
Control	211±29.15
LPS + Stress	86.33±6.159
Hesperidin 50 mg/kg	219.3±25.57
Hesperidin 100 mg/kg	203.0±13.5
Hesperidin 200 mg/kg	165.1±11.37
Diazepam	177.0±14.66

Fig 1: Effect of Hesperidin and Diazepam on Locomotor activity in LPS-Treated Rats by Actophotometer



(Values are mean SEM observation from six animals from each group)
(*p<0.005; **p<0.01; ***p<0.001; ****p<0.0001)

b. Effect of Hesperidin and Diazepam on Elevated Plus Maze Behaviour in LPS-Treated Rats

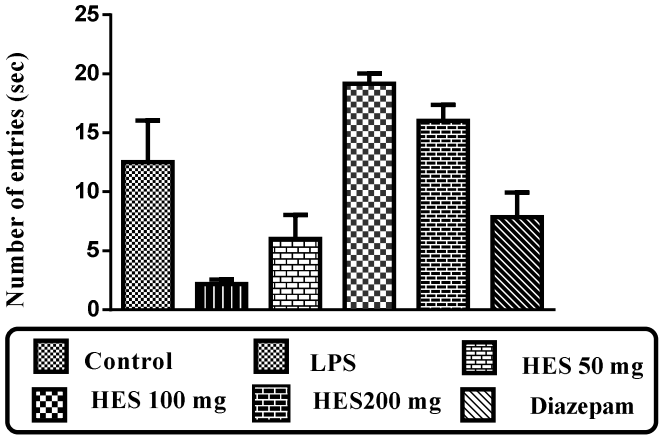
Anxiety levels were determined with a plus-maze apparatus and the results are shown in figure 2 and table 2. When tested on an elevated plus-maze, a significant increase in anxiety levels (time spent in open arms [OP]: 2.16±0.40; time spent in closed arms [CL]:163.84±1.55) were seen with LPS-treated rats on Day 30, when compared with control animals (OP: 12.5± 3.54; CL: 153.6.0±8.34). A significant reduction in anxiety levels were observed with hesperidin treated rats at different doses (50 mg/kg: OP: 6.0±2.22 & CL: 167.1±3.88; 100 mg/kg: OP: 19.16±0.87 & CL: 144.16±5.52; 200 mg/kg: OP: 16.0±1.39 & CL: 149.3±1.68). The behavioral effect of Diazepam was also found to produce significant antianxiety effect (OP: 7.83± 2.12 & CL: 148±6.33).

Table2. Effect of Hesperidin and Diazepam on LPS-Treated by Rats Elevated plus Maze Behavior

Groups	Time Spent In Arms (In Sec)	
	Open (OP) Entry	Closed (CL) Entry
Control	12.5± 3.54	153.6.0±8.34
LPS + Stress	2.16±0.40	163.84±1.55
Hesperidin 50mg/kg	6.0± 2.03	167.1±3.88
Hesperidin 100mg/kg	9.16± 0.87	144.16±5.52
Hesperidin 200mg/kg	16.0±1.39	149.3±1.68
Diazepam	7.83± 2.12	148±6.33

Fig 1: Effect of Hesperidin on Time spent in open & closed arm of EPM in LPS-Treated Rats

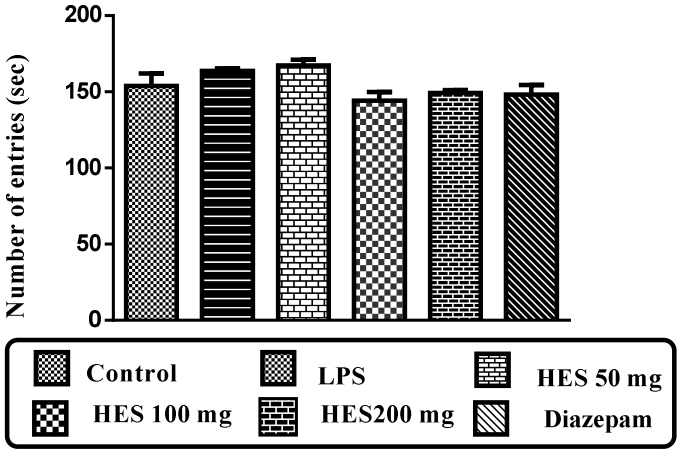
open arm entry



(Values are mean SEM observation from six animals from each group)
(*p<0.005; **p<0.01; ***p<0.001; ****p<0.0001)

Fig 1: Effect of Hesperidin on Time spent in open & closed arm of EPM in LPS-Treated Rats

closed arm entry



(Values are mean SEM observation from six animals from each group)
(*p<0.005; **p<0.01; ***p<0.001; ****p<0.0001)

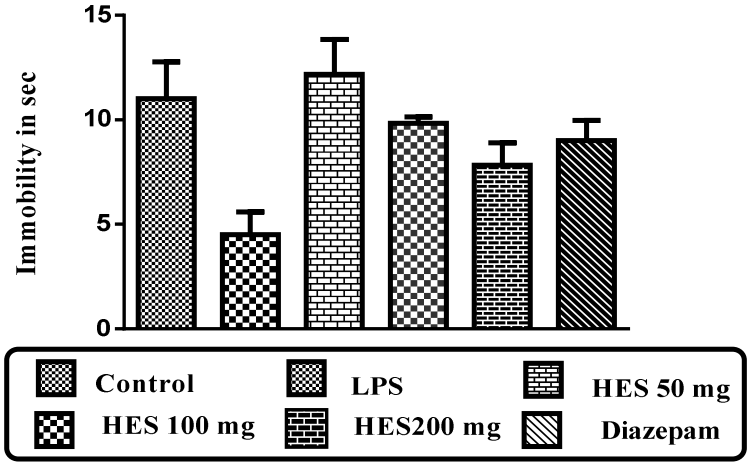
c. Effect of Hesperidin and Diazepam in LPS-Treated Rats in Forced Swim Test

Antidepressant activity of LPS-treated animals was determined by forced swim test and the results are shown in figure 3 and table 3. When tested by forced swim test, a significant decrease in movement level was seen in LPS-treated rats (4.58±1.08), when compared with control animals (11±1.77). A significant increase in movement activity was seen in hesperidin-treated animals at different doses (50mg/kg: 12.16±1.68; 100 mg/kg 9.83±0.30; 200mg/kg: 7.83±1.07). Diazepam (9±0.96) was also found to have significant antidepressant activity.

Table3. Result of effect of Hesperidin and Diazepam in LPS-Treated rats by Forced Swim Test

Groups	Immobility Time (in sec)
Control	11±1.77
LPS	4.58±1.08
Hesperidin 50	12.16±1.68
Hesperidin 100	9.83±0.30
Hesperidin 200	7.83±1.07
Diazapam	9.0±0.96

Fig 1: Effect of Hesperidin on forced swim test in LPS-Treated Rats



(Values are mean SEM observation from six animals from each group)
(*p<0.005; **p<0.01; ***p<0.001; ****p<0.0001)

5.3. Effect of Hesperidin and Diazepam on Rat Brain Antioxidant System in LPS-Treated Rats

5.3.1. Effect of Hesperidin on Superoxide Dismutase (SOD) Levels in LPS-Treated Rat Brain

The SOD profile in the rat brain is depicted in table 4 and figure 4. In comparison to control rats (37.28±1.30), LPS insult resulted in significant reduction of SOD level in brain (75.81±2.79). Pre-treatment with hesperidin at different doses (50 mg/kg: 51.98±0.99; 100 mg/kg: 40.28±0.32; 200mg/kg: 36.87±0.29) for 30 days significantly improve the SOD activity in comparison to LPS-treated groups in the brain studied. Among all the groups, a marked increase in SOD status was observed with 50 mg/kg of hesperidin.

5.3.2. Effect of Hesperidin on Catalase (CAT) Levels in LPS-Treated Rat Brain

Table 4 and figure 4 showed the alteration of CAT levels in brain studied. In comparison to control rats (16.95±0.26), LPS induction resulted in significant reduction of CAT level of brain (2.67±0.39). Pre-treatment with hesperidin at different doses (50 mg/kg: 10.11±0.29; 100 mg/kg: 12.63±0.27; and 200mg/kg: 14.27±0.27) for 30 days significantly improved the CAT activity in comparison to LPS-treated groups in the brain studied. Among all the groups, a marked increase in CAT status was observed with LPS-treated groups administered with 200 mg/kg of hesperidin.

5.3.3. Effect of Hesperidin on Reduced Glutathione (GSH) Levels in LPS-Treated Rat Brain

The effect of hesperidin in LPS-treated rats on GSH levels are summarized in table 4 and figure 5.In comparison to control rats (2.43±0.40), LPS induction resulted in significant increase of GSH level of brain (1.91±0.23). Pre-treatment with hesperidin at different doses (50 mg/kg: 67.29±0.44; 100 mg/kg: 2±0.18; 200mg/kg: 1.80±0.22) for 30 days significantly improved the GSH activity in comparison to LPS-treated groups in the brain. Among all groups, a marked increase in GSH status was observed in LPS-treated groups administered with 50 mg/kg of hesperidin.

5.3.4 Effect of Hesperidin on Malondialdehyde Levels in LPS-Treated Rat Brain

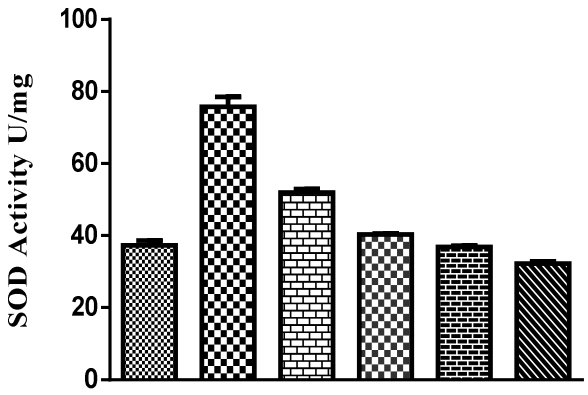
The effect of hesperidin in LPS-treated rats on MDA levels are summarized in table 4 and figure 5.In comparison to control rats (51.39±1.01), LPS induction resulted in significant increase in MDA level of brain (77.28±0.78). Pre-treatment

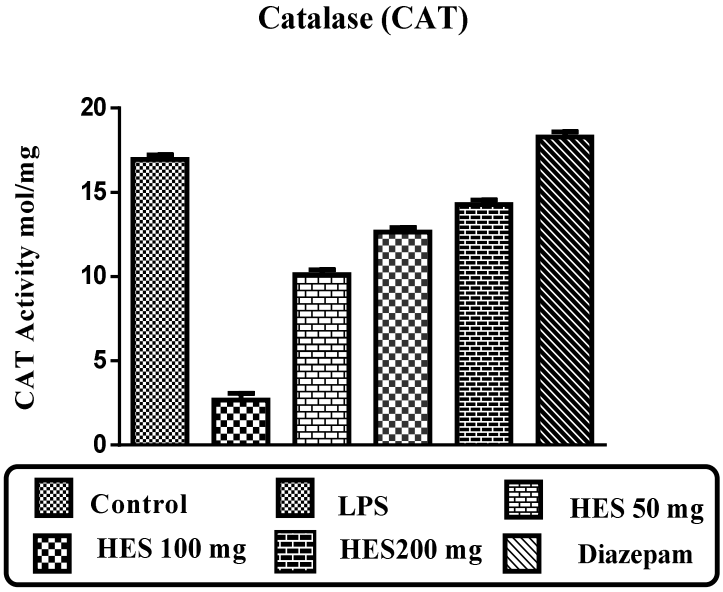
with hesperidin at different doses (50 mg/kg: 67.29±0.44; 100 mg/kg: 59.87±0.45; 200 mg/kg: 48.32±0.47) for 30 days significantly improve the MDA activity in comparison to LPS-treated groups in the brain. Among all groups, a marked increase in MDA status was observed in LPS-treated groups administered with 50 mg/kg of hesperidin.

Table4.Effect of Hesperidin on Antioxidant System in LPS-Treated Rat Brain

Groups	U of SOD/mg protein	µmol of catalase/mg protein	nmol GSH/mg protein	µmol of MDA/mg protein
Control	137.28±1.30	16.95±0.26	2.43±0.40	51.39±1.01
LPS + Stress	75.81±2.79	2.67±0.39	1.91±0.23	77.28±0.78
Hesperidin 50 mg/kg	51.98±0.99	10.11±0.29	1.67±0.19	67.29±0.44
Hesperidin 100 mg/kg	40.28±0.32	12.63±0.27	2.0±0.18	59.87±0.45
Hesperidin 200 mg/kg	36.87±0.29	14.27±0.27	1.80±0.22	48.32±0.47
Diazapam	32.33±0.57	18.29±0.29	1.64±0.01	47.99±0.44

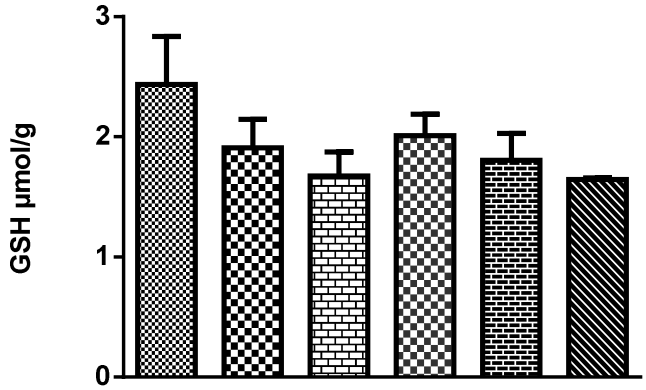
Fig 1: Effect of Hesperidin on Superoxide Dismutase (SOD) and Catalase Activity in LPS-Treated Rats

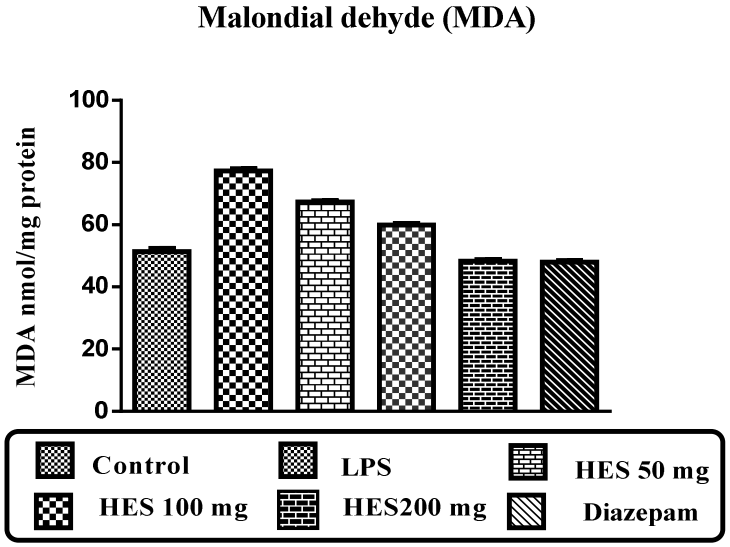




(Values are mean SEM observation from six animals from each group)
(*p<0.005; **p<0.01; ***p<0.001; ****p<0.0001)

Fig 1: Effect of Hesperidin on Glutathione (GSH) and Malondial dehyde (MDA) Activity in LPS-Treated Rats





(Values are mean SEM observation from six animals from each group)
(*p<0.005; **p<0.01; ***p<0.001; ****p<0.0001)

5.3.5. Effect of Hesperidin on Brain Phospholipid Levels in LPS-Treated Rats

The effect of hesperidin in LPS-treated rats on brain phospholipids levels are summarized in table 5 and figure 6. In comparison to control rats (69.51±0.56), LPS stress resulted in significant decrease of phospholipids level of brain (34±0.41). Pre-treatment with hesperidin at different doses (50 mg/kg: 42.97±0.27; 100 mg/kg: 56.15±0.39; 200 mg/kg: 61.74±0.28) for 30 days significantly improved the phospholipid level in comparison to LPS-treated groups in the brain. Among all groups, a marked increase in phospholipid level was observed in LPS-treated groups administered with 200 mg/kg of hesperidin.

5.3.6. Effect of Hesperidin on Brain Cholesterol Levels in LPS-Treated Rats

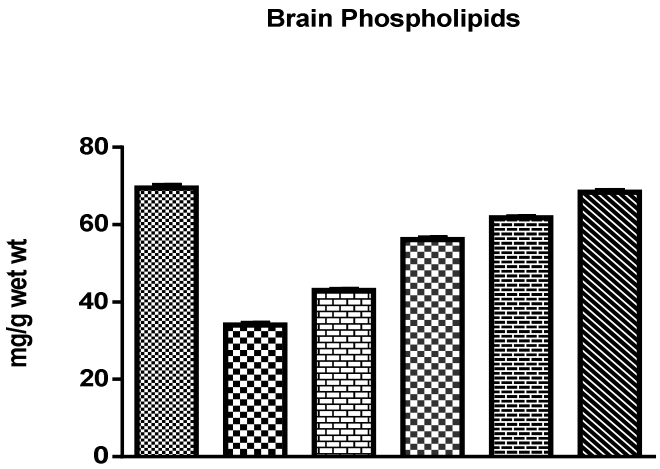
The effect of hesperidin in LPS-treated rats on brain cholesterol levels are summarized in table 5 and figure 6. In comparison to control rats (22.05±0.27), LPS stress resulted in significant decrease of cholesterol level of brain (14.22±0.30). Pre-treatment with hesperidin at different doses (50 mg/kg: 18.10±0.28; 100 mg/kg: 18.95±0.29; 200 mg/kg: 19.27±0.35) for 30 days significantly improve the cholesterol

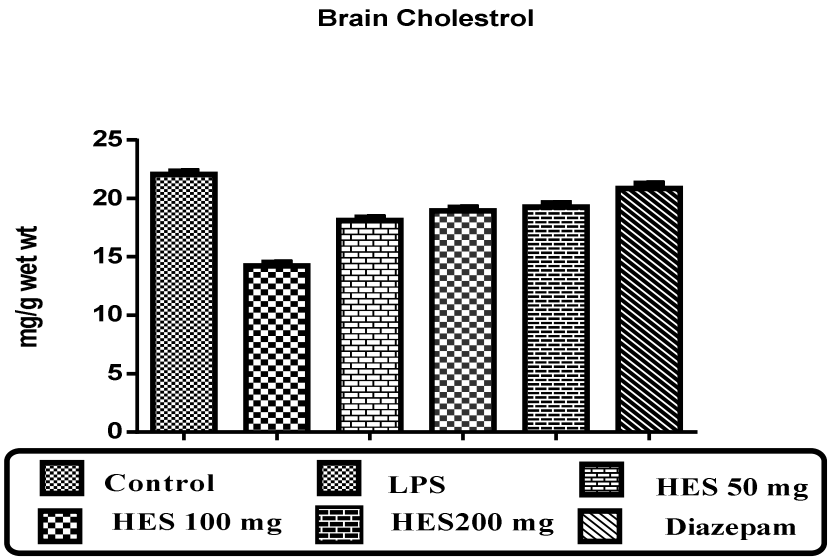
level in comparison to LPS-treated groups in the brain. Among all groups, a marked increase in cholesterol level was observed in LPS-treated groups administered with 200 mg/kg of hesperidin.

Table5. Summary of Effect of Hesperidin on Brain Phospholipid and Cholesterol Levels in LPS-Treated Rats

Groups	Brain phospholipids mg/kg	Brain cholesterol mg/g
Control	69.51±0.56	22.05±0.27
LPS + Stress	34.0±0.41	14.22±0.30
Hesperidin 50 mg/kg	42.97±0.27	18.10±0.28
Hesperidin 100 mg/kg	56.15±0.39	18.95±0.29
Hesperidin 200 mg/kg	61.74±0.28	19.27±0.35
Diazepam	68.38±0.35	20.86±0.42

Fig.6 Effect Of Hesperidin On Brain Phospholipids (BP) And Cholestrol Levels in LPS Treated Rats





(Values are mean SEM observation from six animals from each group)
(*p<0.005; **p<0.01; ***p<0.001; ****p<0.0001)

5.4.1. Histological changes in LPS + Stress Treated Rat Brain

The histological changes of brain sections of rats treated with LPS and stress, hesperidin and diazepam are shown in **images 1-12.**

Normal cerebral brain parenchyma (**image 1)** and normal cerebral molecular layer (**image 2)** are seen in the brain sections of control rats. But, acute neuronal injury (**image 3)** and neuronal swelling (**image 4)** are seen in LPS+stress treated rats.

In rats treated with hesperidin (50 mg/kg), section from brain showed normal brain parenchyma (**image 5)** and focal acute neuronal injury (**image 6)**.

In rats treated with hesperidin (100 mg/kg), section frombrain showed no perivascular lymphocytic infiltration/microcystic formations in glial cells (**image 7)** and mild astrocytes hypertrophy (**image 8).**

No pathological brain parenchyma (**image 9)** and normal molecular layer (**image 10)** are shown in brain sections of rats treated with hesperidin (200 mg/kg).

Brain sections of rats treated with diazepam showed normal brain parenchyma (**image 11)** and acute neuronal injury **(image 12)**.

The result showed that the endotoxin LPS-caused brain damage, are prevented by hesperidin at higher doses (100 mg/kg and 200 mg/kg). Hesperidin at lower doses (50 mg/kg) is found to be less effective.

Image1. Haematoxylin eosin-stained brain sections of control rats (Normal brain parenchyma, 10x)

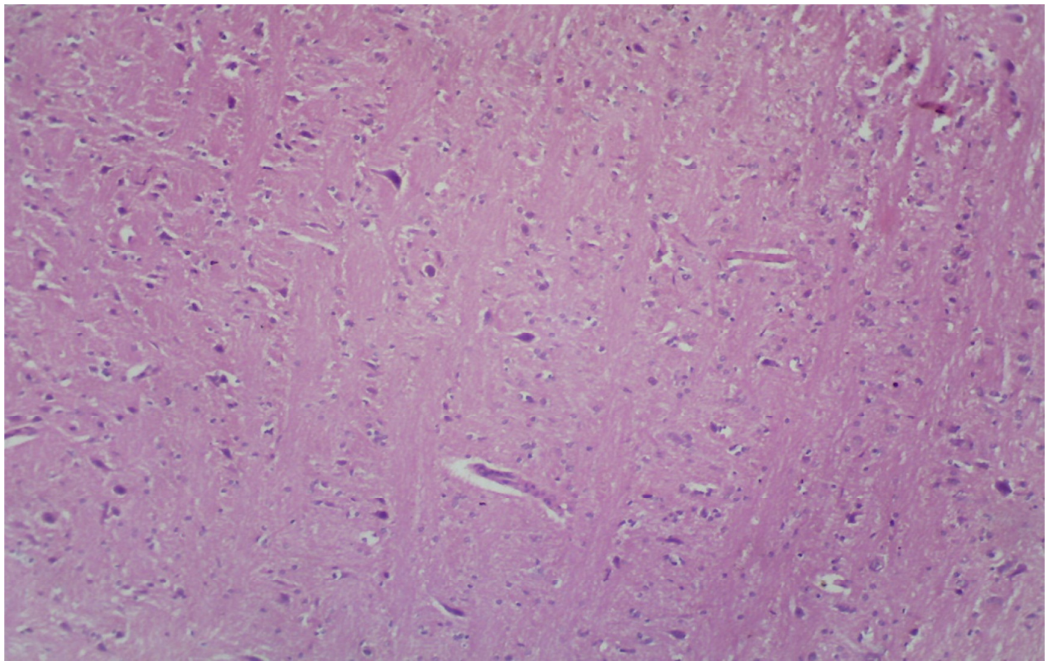


Image2. Haematoxylin eosin-stained brain sections of control rats, 40x)

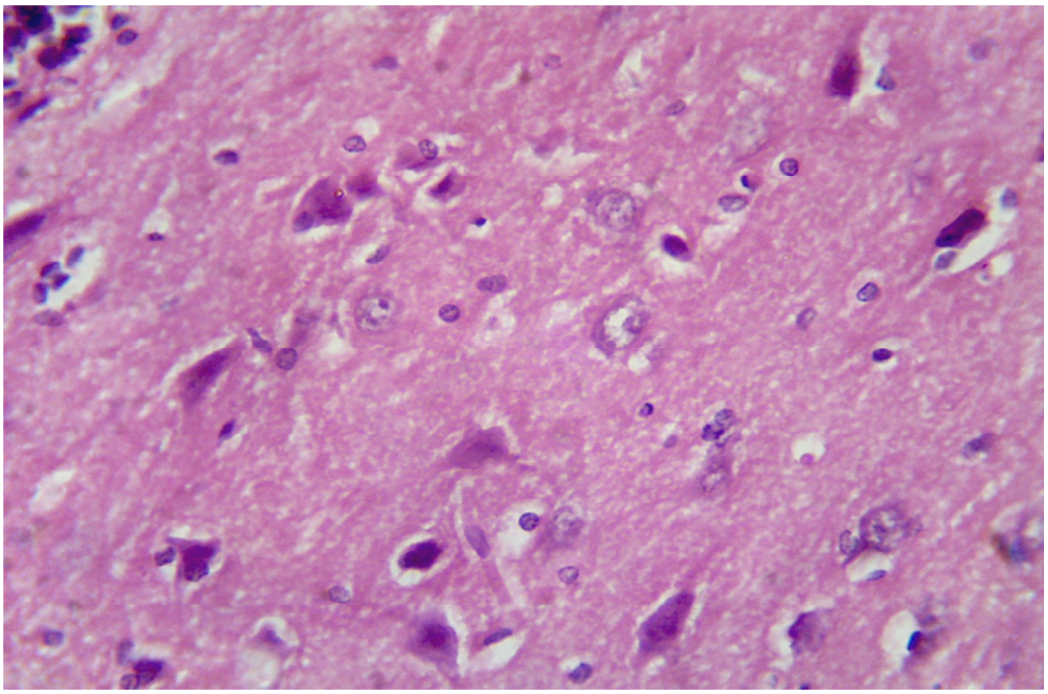


Image3. Haematoxylin eosin-stained brain sections of LPS treated rats (Acute neuronal injury, 10x)

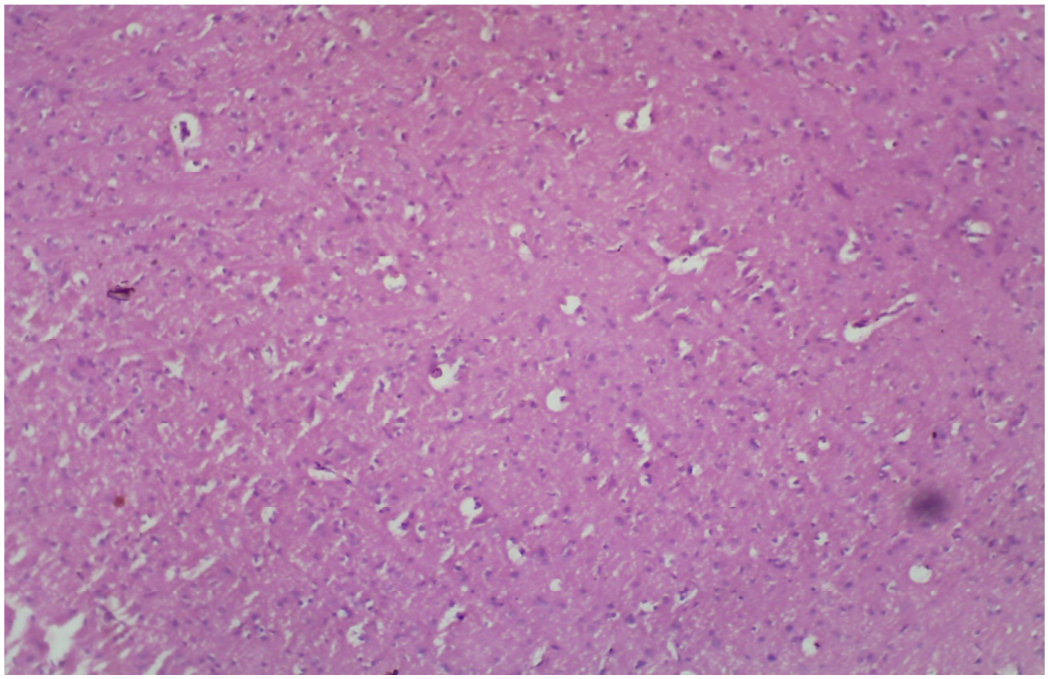


Image4. Haematoxylin eosin-stained brain sections of LPS treated rats (Neuronal swelling, 40x)

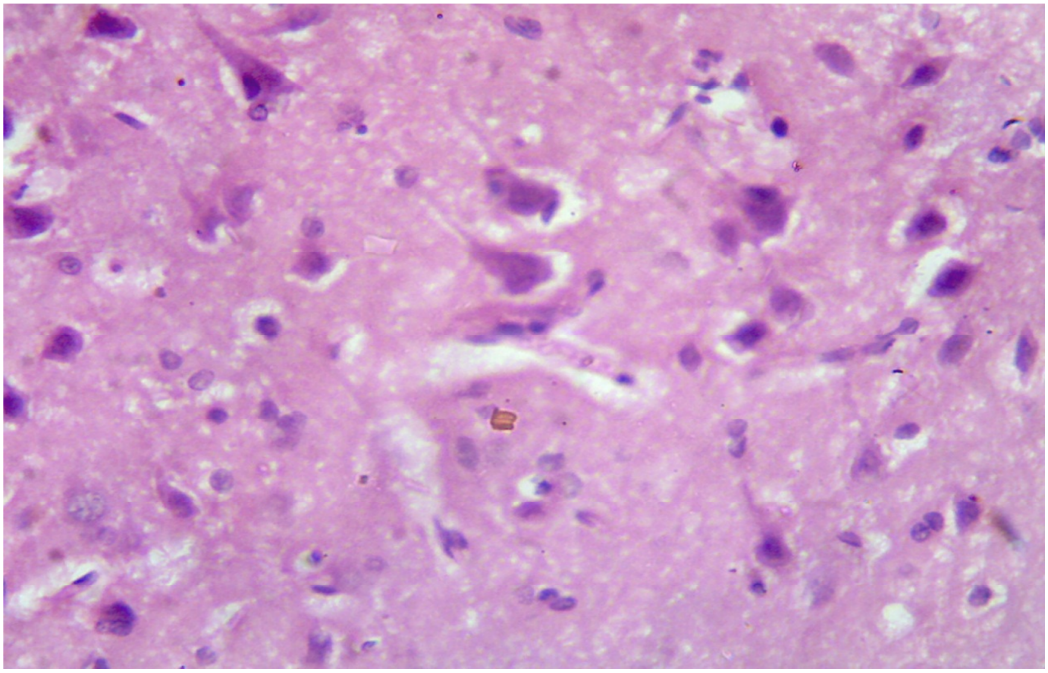


Image5. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 50 mg/kg (Normal brain parenchyma, 10x)

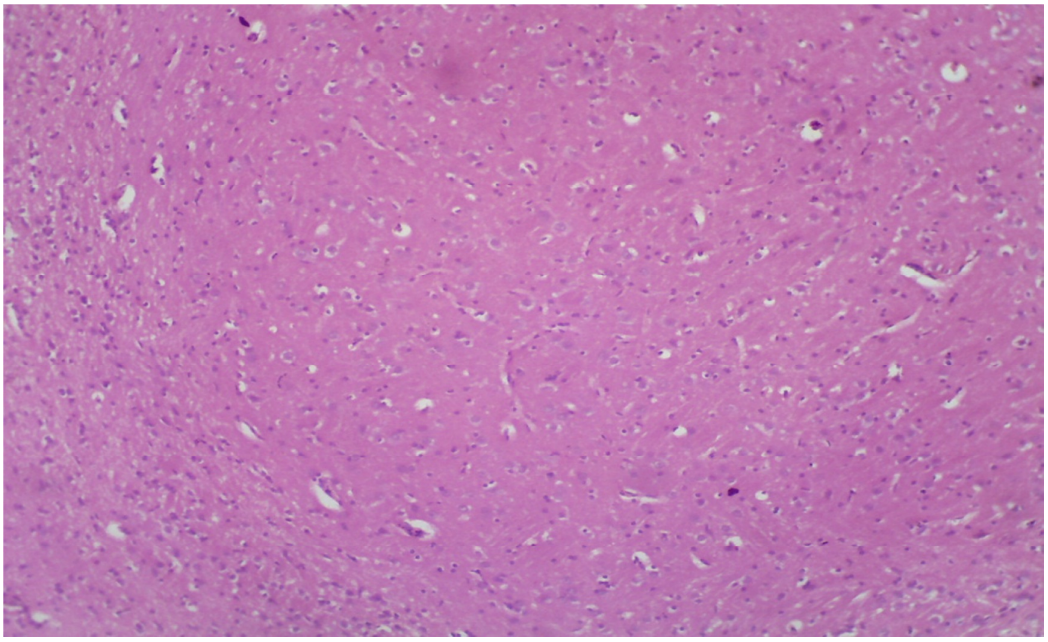


Image6. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 50 mg/kg (Focal acute neuronal injury, 40x)

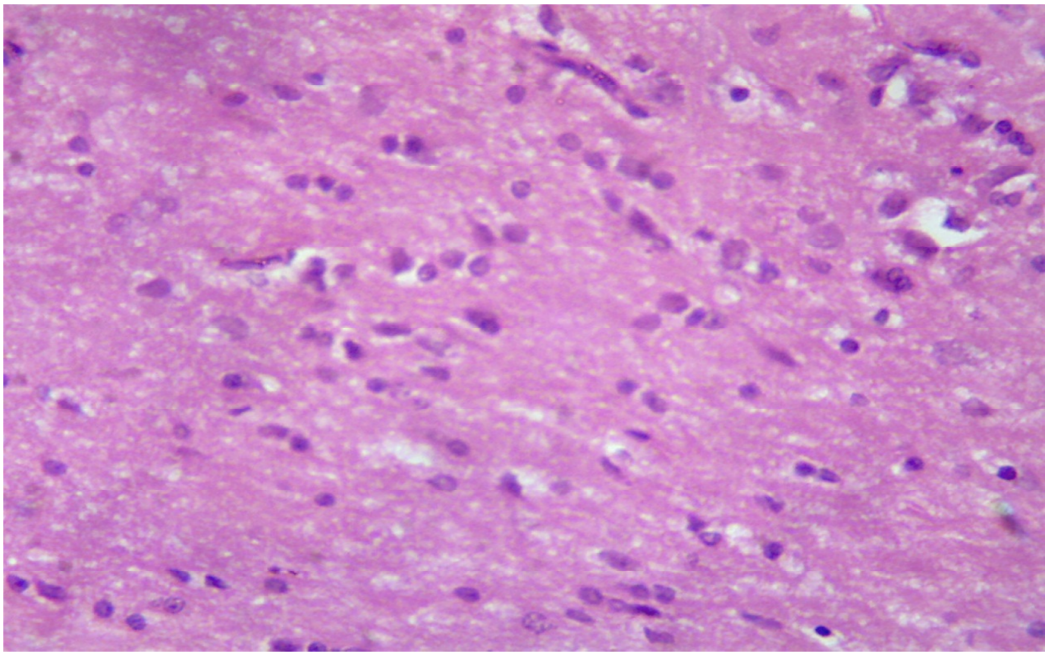


Image7. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 100 mg/kg (40x)

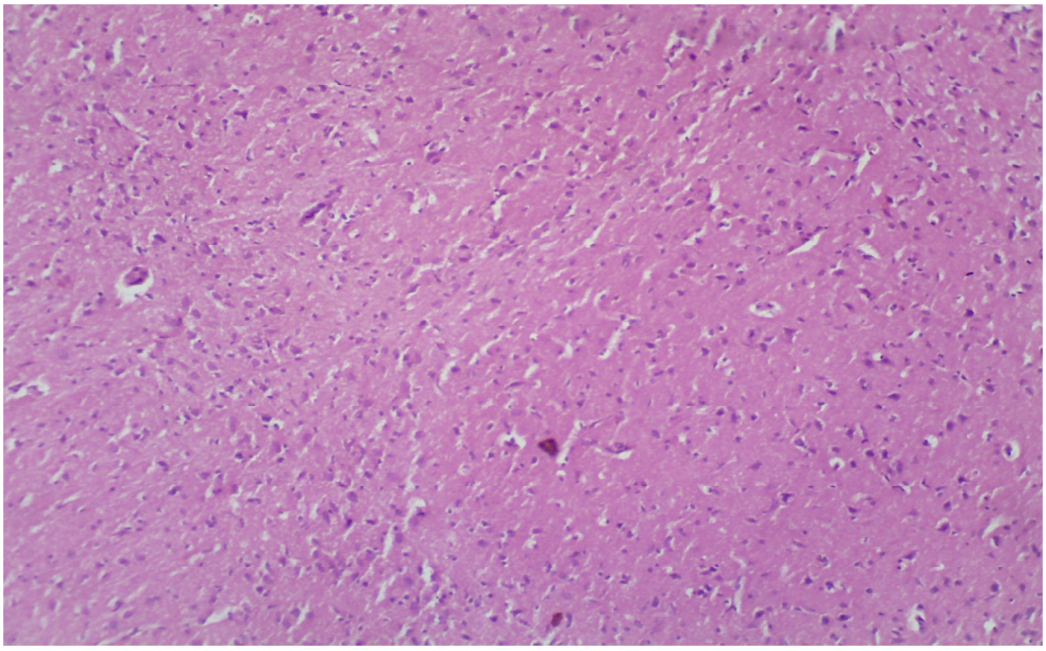


Image8. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 100 mg/kg (Mild astrocytes hypertrophy, 40x)

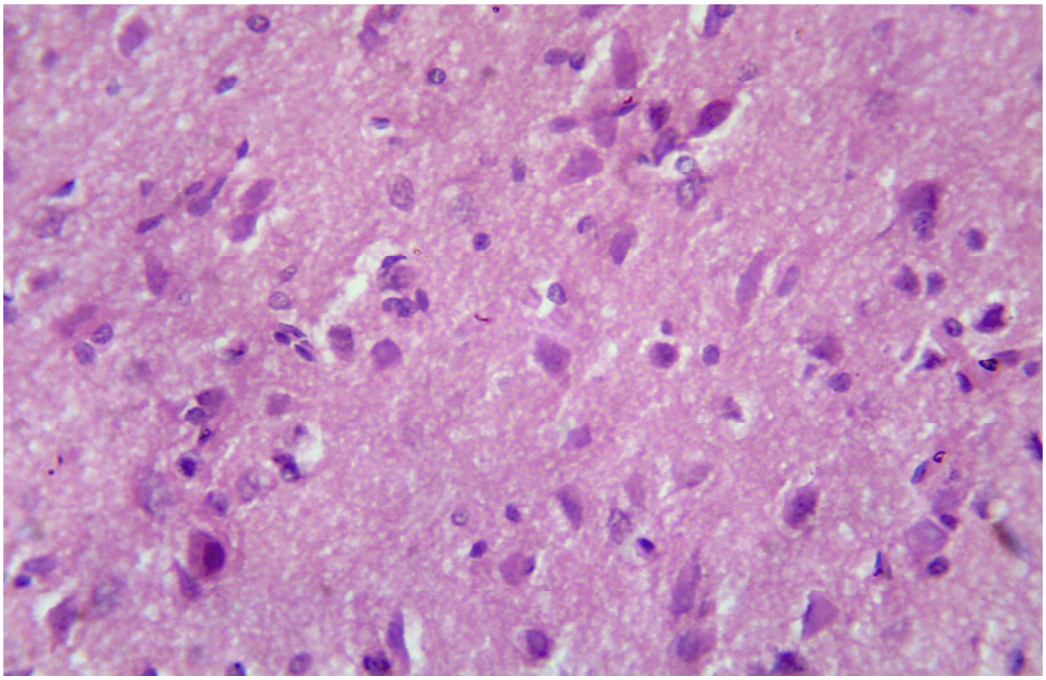


Image9. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 100 mg/kg (10x)

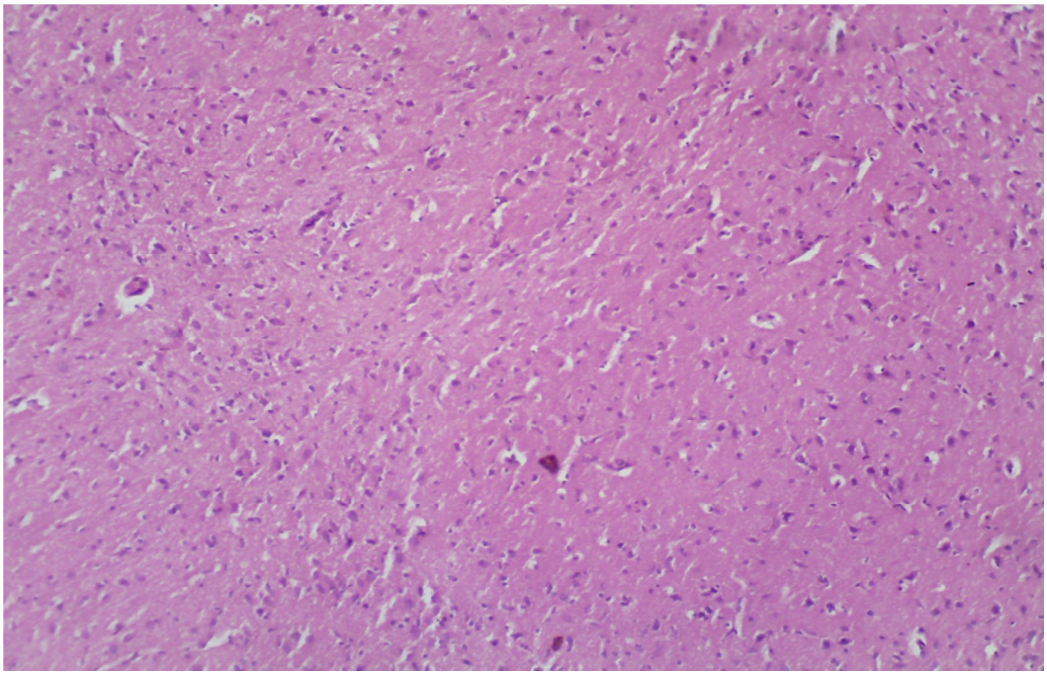


Image10. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 100 mg/kg (40x)

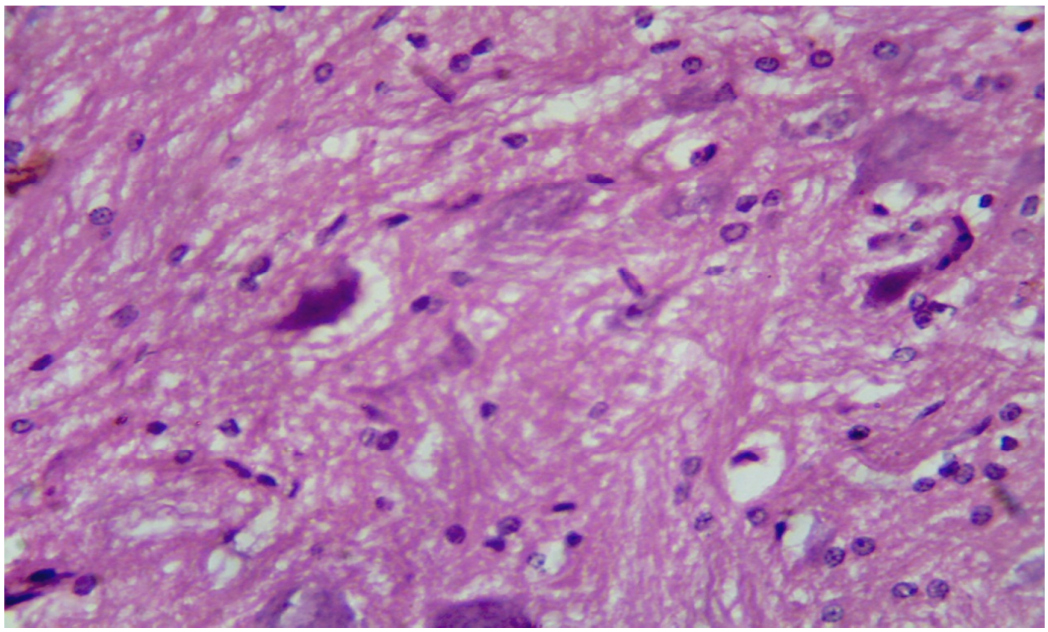


Image11. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 200 mg/kg (10x)

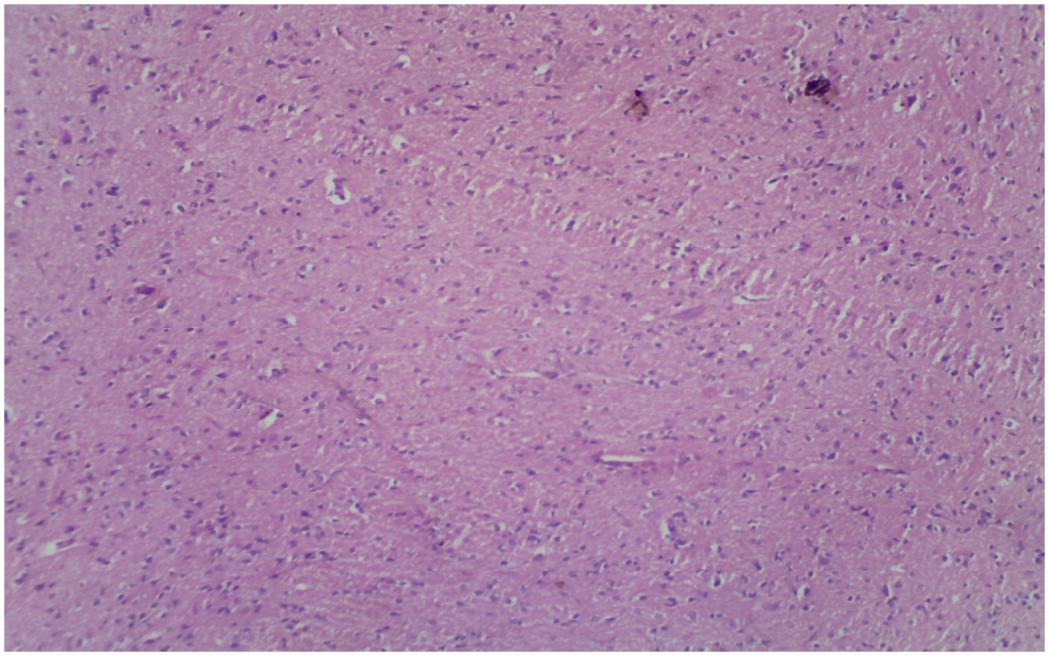
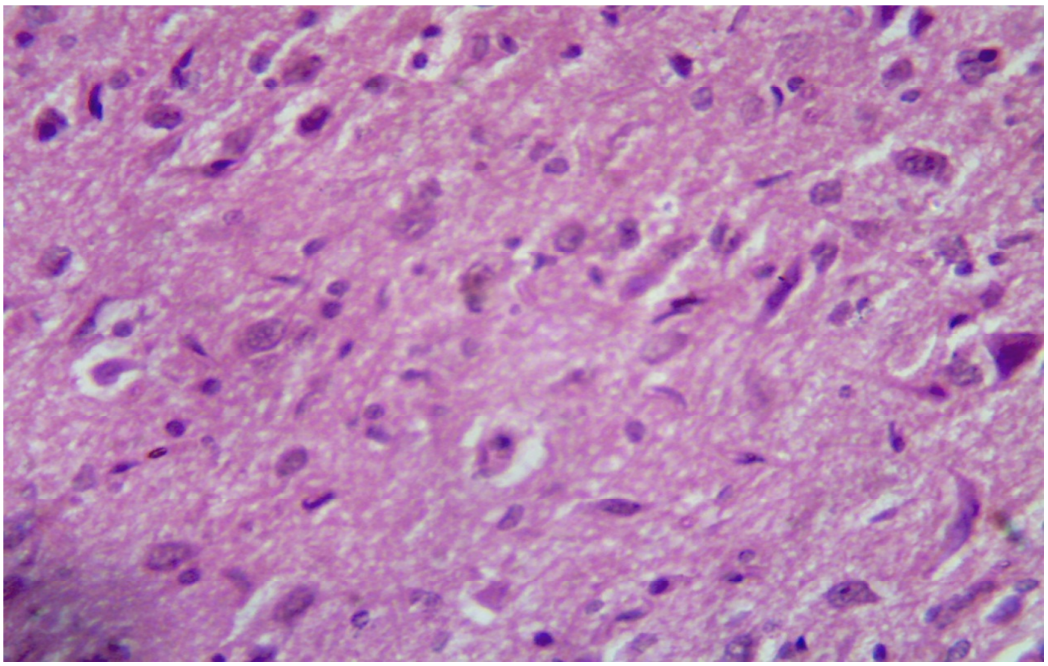


Image12. Haematoxylin eosin-stained brain sections of rats pre-treated with hesperidin 200 mg/kg (40x)



7. DISCUSSION

The present study was designed to evaluate the neuroprotective effect of hesperidin against oxidative stress induced by LPS endotoxemic and acute stress (forced swim-induced stress) in SD rats. The neuroprotective effect was assessed using a battery of general behavioral tests, biochemical analysis and histopathological examination.

In an experiment conducted previously at this research lab, it was observed that hesperidin was found to protect the brain against LPS-induced endotoxemia in SD rats. The findings of the previous study encouraged us to study the potential protective effect of hesperidin against acute stress in addition to subjecting it to LPS induced endotoxemia.

Endotoxemia was induced in rats using a single intraperitoneal injection of LPS (1.5 mg/kg body weight). Following LPS administration, the animals were subjected to acute stress (forced swim-induced stress).

Neurodegeneration is always associated with perturbations in the levels of brain enzymatic and non-enzymatic antioxidant system in stressful conditions. Hence, the effects of hesperidin on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and MDA and as an index of lipid per oxidation (LPO), brain phospholipids and cholesterol were analyzed in rat brains.

In addition, a histopathological examination of the rat brain, stained in haematoxylin and eosin was performed to evaluate the neuroprotective role of hesperidin pre-treatment against LPS + acute stress-induced neurodegeneration in rats.

Lipopolysaccharide (LPS), a glycolipid component of the cell wall of gram-negative bacteria, plays a major role in the etiology of infection-associated inflammatory reactions and septic shock *(Galanos & Freudenberg, 1993; Ulevitch & Tobias, 1995).* Activation of immune system in response to either infection or LPS produces neurophysiological, neuroendocrine and physiological changes. Besides these neuroendocrine effects, LPS induced changes in the levels of biogenic amines in the hypothalamus are without doubt, significant contributors to the other central effects of LPS such as behavioral parameters, anxiety depression and cognition deficits.

6.1 On General behavioral parameters

The findings of our experiment revealed that exposure to LPS + acute stress resulted in increase in body temperature of animals in all the groups. Furthermore, no increase in body temperature was observed in indomethacin treated group. This finding compliments work showing that the NSAIDs attenuated decrease in body weight and sickness behavior induced by LPS *(De La Garza, 2004; Okamoto, 2002; O'Reilly et al., 1988; Valles et al., 2000; Yirmiya, 1996).*

The decrease in locomotors activity observed with LPS-treated rats can be correlated to similar reported findings in male LPS-treated mice *(Engeland et al., 2001).* Treatment with hesperidin attenuated the depressed general behavior parameters, effects on body temperature, in a dose dependent manner.

The suppression of food and water intake, body weight and body temperature is considered to be symptoms associated with depression and termed as 'sickness behavior'. The sickness behavior is in some ways similar to depressive behavior following LPS treatment.

Barnum et al., (2012), Filho et al., (2013) and Souza et al., (2013) have reported on the antidepressant action of hesperidin and further suggested that antidepressant drugs are clinically effective after chronic administration, Similarly, in the present study, chronic hesperidin treatment (for 30 days), may have produced adaptive changes in several neural systems, particularly monoaminergic systems and the hypothalamus-pituitary-adrenal axis (HPA axis) *(Parrott et al., 1995; Lenczowski et al., 1997; Rivier et al., 1989; Tilders et al., 1994),* causing attenuation of LPS-induced suppression of food and water consumption and body weight gain, hyperthermia and general behavior parameters of the rats. These findings provide further support for the similarity between LPS-induced immune activation, anxiety and depression. *(Filho et al.,2012).*

Activation of the HPA axis alters neurotransmitter, immune, and behavioral functions and may contribute to the development of depressive symptoms in humans *(Lenczowski et al., 1997; Rivier et al., 1989; Tilders et al., 1994; Pariante & Miller, 2001; Raison & Miller, 2001).* In a report, *De La Garza et al., (2004)* showed that LPS administration + stress (*Gadek-Michalska et al., 2005*) strongly increased corticosterone release affecting behavioral parameters, which was significantly lower

in diclofenac-treated rats and, LPS induced changes in the concentrations of biogenic amines in the hypothalamus are without a doubt significant contributors to the other central effects of LPS such as anorexia, sleep and fever (*Bluthé et al., 1992; Klir et al., 1993; Kluger, 1991; Nava et al., 1997; O'Reilly et al., 1988; Yirmiya, 1996).* Thus hesperidin is considered to antagonize the effects of LPS on HPA axis, thereby normalizing the behavioral parameters observed in this study.

Our findings on the levels of plasma corticosterone following LPS and stress are substantiated by the reports of *Gadek-Michalska et al., 2005*. Hesperidin has been found to prevent stress-induced increase in plasma corticosterone (*Takahashi et al., 1998*), and normalize the stress-induced changes in biogenic amines like NA, Adr, 5-HT, 5-HT (*Goldie et al., 1982),* turnover in brain following. Hence, the inhibition of rise in plasma corticosterone and its effect of central neurotransmitter levels by hesperidin, in response to several stressors may be considered to be responsible for normal behavioral parameters observed with hesperidin-treated animals.

PGE_2 levels of brain interstitial fluid rise following peripheral injection of LPS + stress (*Gadek-Michalska et al., 2005*). Pharmacological blockade of PGE_2 synthesis attenuates many peripheral LPS-induced responses, such as fever *(Sehic et al., 1996),* brain *c-fos* expression, HPA-axis activation *(Parrott et al., 1995),* increased splenic sympathetic activity *(MacNeil et al., 1997),* activation of serotonergic and noradrenergic neurotransmission in hippocampus *(Linthrost et al., 1996),* and increased BBB permeability *(de Vries et al., 1996).* Increased production of PGE_2 in brain, therefore, is critically involved in these CNS-linked responses to peripheral LPS on behavioral parameters. Hence, the positive behavioral effects produced by hesperidin may be attribute to its ability to inhibit and down regulate AA metabolites, PGE_2 and COX activity, peripherally *(Mura et al., 1998),* Another possible explanation of the present result is that, chronic treatment with hesperidin suppressed LPS-induced activation of cytokine systems, which normally mediate the behavioral effects of LPS.

In summary LPS produced significant locomotor decrements and other general behavior parameters more robustly. In addition, LPS caused significant changes in body temperature. The behavioral effects were significantly attenuated by hesperidin in a dose dependent manner. The changes in body weight, food and water intake and hyperthermia caused by LPS were all significantly restored by hesperidin.

Biochemical studies

All cells in the body are exposed chronically to oxidants from both endogenous and exogenous sources but are also equipped with an antioxidant system. Reactive oxygen and nitrogen species, if unchecked, can contribute to chronic disease development by ox datively modifying lipids, nucleic acids and proteins *(Liu et al., 1996),* Of all the organs, the brain is thought to be most vulnerable to oxidative damage due to high oxygen consumption, presence of high levels of PUFA and non-degenerative nature of neurons, leading to various neurodegenerative diseases *(Floyd & Carney, 1992),* making them the primary target for different stressors that initiate lipid peroxidation, a self-propagating chain reaction resulting in significant tissue damage and disease.

Under normal conditions, brain microglias are involved in immune surveillance and host defense against infectious agents. Activation of the immune system in response to infection produces neural, neuroendocrine, and behavioral effects. However, microglia readily become activated in response to injury or immunological challenges, as indicated by a change in morphology from a ramified resting state to an amoeboid appearance with an increase in the expression of major histocompatibility complex (MHC) molecules and complement type 3 receptor *(Graeber et al., 1994; Kaur & Ling, 1992; Kreutzberg, 1996; Streit et al., 1988).* Endotoxin-mediators induced activation of microglia is believed to contribute to neurodegenerative process through the release of proinflammatory and/or cytotoxic factors, including IL-1ß,IL-6, TNF-α, INF-γ, induction of NF-κB, macrophage inflammatory protein (MIP), NO, ROS, histamine, proteases, neuropeptides, quinolinic acid and AA metabolites *(Brosnan et al., 1994; Chao et al., 1992; Dickson et al., 1993; Espey et al., 1997; Lee et al., 1993; Matsuo et al., 1995).* Among the AA metabolites, prostaglandin E_2 (PGE_2) levels of brain interstitial fluid was found to rise following peripheral injection of LPS (Sehic et al., 1996). Pharmacological blockade of PGE_2 synthesis attenuates many peripheral LPS-induced responses, such as fever *(Sehic et al., 1996)*, brain c-*fos* expression, HPA axis activation *(Parrott et al., 1995),* increased splenic sympathetic activity *(Gaykema et al., 1995; MacNeilet al., 1997),* activation of 5-HT and NA neurotransmission in hippocampus *(Linthorstet al., 1996*), and increased BBB permeability *(de Vries et al., 1996).*

Increased production of PGE_2 in brain, therefore, is critically involved in the CNS-linked responses to peripheral LPS. There are three enzymes that are essential for the production of PGE_2: PLA_2, COX, and PGE_2isomerase. COX-1 levels are relatively insensitive to inflammatory stimulation. COX-2, on the other hand, is strongly induced by inflammatory factors such as LPS *(DeWitt, 1991).* The induced expression of COX-2 in the brain is thought to play an important role in the elevation of central PGE_2 levels in response to peripheral LPS *(Breder et al., 1995; Cao et al., 1995).* Over expression of COX-2 has been found in the AD brains and a number of epidemiological studies have indicated that NSAIDs, inhibitors of COX, are beneficial for AD patients in delaying the clinical progression *(Zandi & Breitner, 2001).*

Several previous reports have demonstrated a number of antioxidant and anti-inflammatory activity as due to its COX and LOX inhibitory potential of hesperidin and researchers have associated these effects to the presence of phenolic compounds in hesperidin *(Hafida oufedjikh et al 1999).* Hesperidin has also been reported to possess significant anti-inflammatory activity against a numbers of mediators of inflammation, in particular, against PGE_2, leukotirenes (LT) and AA-induced paw edema in rats *(Baroody et al., 2000)* by virtue of their capacity to block both cyclooxygenase *(Raymond et al., 1998).*

Several lines of investigations now suggest that primary action of LPS in the brain may be mediated by an increase in the concentrations of pro-inflammatory cytokines and several autacoids factors *(Hopkins & Rothwell, 1995; Nava et al., 1997).*

It is well known that intense stress response results in the generation of ROS [hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide anion radical (O2•)], that cause lipid peroxidation; especially in membranes and can play an important role in tissue injury.

In this study the lipid peroxidation levels increased markedly following LPS treatment, which is in agreement with the findings of (Abd El-*Gawad & Khalifa, (2001).*In order to neutralize ROS, the body uses enzymatic copper-, zinc-SOD, CAT and selenium dependent GSHPx and non-enzymatic (reduced glutathione) antioxidants *(Samson et al, 2007).*

The increase in the levels of SOD, and CAT observed following LPS exposure is an indicator of a relative increase in the superoxide radical production. The increased SOD activity is therefore an indication that the brain's antioxidant machinery is activated in response to excessive generation of free radicals. Enhanced SOD activity catalyzes the conversion of superoxide anions to H_2O_2 which in turn could stimulate the second line of defense which includes GSHPx and CAT. These enzymes convert H_2O_2 into water and molecular oxygen, rationalizing the cause for the elevation of these two during the noise stress. CAT levels are also elevated in all the regions in acute noise exposure. This indicates that CAT and GSHPx substitute for each other in different brain regions.

The significant reduction in GSH levels in LPS exposed animals may be justifiable as due to increased production of free radicals. Furthermore, it is also suggests that the ratio of reduced/oxidized glutathione in the cell is a good indicator of the level of oxidative stress *(Abd El-Gawad & Khalifa, 2001).* Therefore, the significant decrease in the GSH observed in this study indicates the oxidative stress in discrete regions of brain due to LPS exposure. The glutathione status of a cell could be taken as the most accurate single indicator of the health of the cell as the GSH depletion determines the vulnerability to oxidant attack. Animals exposed to LPS-stress showed depletion of GSH level in brain while treatment with hesperidin attenuated this depletion, possibly by reducing oxidative stress-induced generation of ROS.

The enhanced production of brain TBARS observed in our study by LPS injection is in agreement with the *in vivo* study of *Mustafa et al, 1994* and *in vitro* study of *Sewerynek et al., (1995).* Several mechanisms were postulated to explain this phenomenon. One depends on the enhanced release of cytokines that promote the formation and release of ROS and NO from microglia cells *(Woodroofe et al., 1995).* Another mechanism is based on the release of excitatory amino acids (EEAs), aspirate and glutamate that induce free radical formation during their physiological action *(Moghaddam et al., 1993).* These results may be interpreted through microglia activation associated with the production of NO and ROS in response to glutamatergic stimulation *(Garthwaite et al., 1989).* A third mechanism is related to the LPS-induced mobilization of mitochondrial calcium, which in turn, activates the AA cascade that produces ROS *(Richter & Kass et al., 1991).* It has been found that LPS

stimulates the production of Ca^{2+}-independent NOS with different time courses in various tissues *(Grandati et al., 1997).* Regardless of the source of NO, high levels of NO have been associated with membrane lipid peroxidation *(Radi et al., 1991).* The fall in brain GSH content following LPS injection is supported by a similar study *(Mostafa et al, 1994).* The increased oxidative stress depletes cellular stores of brain antioxidants such as GSH and vitamin E *(Thomas, 1994).*

The over production of free radicals can be detoxified by the endogenous antioxidants causing their cellular stores to be depleted *(Thomas, 1994).* This is in accordance with our results as manifested by diminishing GSH content in shocked rats. GSH depletion has been found to dramatically increase cellular sensitivity toward NO, suggesting that intracellular GSH pools act to scavenge NO or NO-derived species *(Walker et al., 1995).* Reduced brain GSH may also be due to reduced synthesis as a result of energy blockade since NO irreversibly inhibits complexes I–III of the mitochondrial electron-transport chain and cytochrome c-oxidase inhibiting cellular respiration and ATP production *(Lizasoain et al., 1996).* Shock was also associated with elevated brain GSHPx activity that acts as a crucial enzymatic defense mechanism against hydrogen peroxide and organic peroxides. Previous reports describing the response of SOD to different types of oxidative stresses were conflicting. Our results are in agreement with a number of reports indicating mild increase of no change in brain SOD levels following LPS-induced endotoxemic stress *(Paschen et al., 1985; Heap et al., 1995).* Measuring SOD activity in whole brain homogenate may not reflect specific localized changes in the activity of this enzyme since it varies significantly between different brain regions *(Fishman et al., 1987).*

In this respect, it is worthy to note that a brain antioxidant system is the main target of ROS toxicity due to its high oxygen consumption *(Skaper et al., 1999).* The free radical mediated lipid peroxidation has been proposed to be critically involved in many neurological disorders and in the degenerative process associated with stress *(Arivazhagan et al., 2001). Manoli et al., 2000, and Baek et al., 1999,* concluded that the vulnerability to oxidative stress in the brain is region specific and is dependent upon local endogenous iron content and their ability to produce lipid peroxides. *(Mandavilli & Rao et al., 1996* reported that regions like cortex, hypothalamus, hippocampus and striatum are more susceptible to oxidative damage when compared to cerebellum. In this study, we have observed that cerebellum, was also equally

susceptible to oxidative damage induced by LPS. This is also in agreement with previous conflicting report by *(Abd El-Gawad & Khalifa, 2001).* Increase in TBARS and decrease in the activity of CAT, and GSH, observed in the rat brain following LPS treatment, were similar to that of earlier reports, wherein different stressors have been reported to induce similar changes in these parameters *(Bhattacharya et al., 2001).*

On the basis of published reports, and from our study, we can speculate that LPS-induced effects of indomethacin, used as a standard drug in this model, involve alterations of neuroendocrine, neuroimmune, and neurochemical function *(Gaykema et al., 1995; Linthrost et al., 1996)* and that NSAIDs counteract stress hormone *(Dunn & Welch, 1991; Okamoto, 2002)*, pro-inflammatory cytokine release, and neurochemical effects *(De La Garza et al., 2004)* produced by endotoxin or cytokine exposure. The observed neuroendocrine and behavioral effects produced by NSAIDs may be the result of its actions as a COX-1 inhibitor. The effects produced by indomethacin may also result from its actions on PGE_2, which is a major COX product at inflammatory sites. In a recent study, LPS-induced increases in PGE_2 production were significantly attenuated by diclofenac, but not by a COX-2-selective inhibitor *(Giuliano & Warner, 2002).* Future studies should address NSAID selectivity (a comparison of non-selective COX inhibitors vs. COX-2 inhibitors). Overall, these findings compliment emerging data that implicate a novel role for NSAIDs in the treatment of depressive-like behavior or symptoms associated with neuroendocrine or neuroimmune dysfunction *(Linthrost et al., 1996; Anisman & Merali, 2002; De La Garza et al., 2004).*

In addition, phenolic flavonoids present a strong affinity for iron ions, which are known to catalyze many processes leading to the appearance of free radicals *(Kaneko et al., 1994).* It is well established that LPS induces protein tyrosine phosphorylation *(Novagrodsky et al., 1994).* The phosphorylation is an early event of the LPS-induced cytotoxicity and results in the production of inflammatory cytokines *(Arditi et al., 1995).* Therefore, LPS-induced cytotoxicity could be prevented by tyrosine kinase inhibitors. The antitoxic and organ protective effects of flavonoids *(Middleton & Kandaswami, 1993)* and their inhibitory activity against tyrosine protein and serine/threonine protein kinesis *(Hagiwara et al., 1988)* may provide a plausible explanation for the protective action of hesperidin in LPS-induced shock.

Oral hesperidin treatment produced significant brain protection as evidenced by reduced TBARS to the basal level, attenuating CAT production and restoring the GSH activity.

The observation that an insignificant alteration in cholesterol and phospholipids levels, which usually affected membrane fluidity has to be contributed by analysis of ATP/ADP ratio and NA+,K+-ATPase activity. Therefore, such minimal changes in the levels of cholesterol and phospholipids in isolation is thus an insufficient indicator on the membrane fluidity in rat brains.

In conclusion, hesperidin alleviated anxiety in locotmotor, elevated plus maze and forced swim tests, the studied antioxidant pretreatments were effective in reducing LPS-induced brain oxidative stress as evidenced by quenching of free radicals and elevation of the antioxidant GSH. All treatments elicited significant protection but hesperidin 200 mg/kg produced the best results.

Neither LPS nor antioxidants had an impact on brain phospholipids or cholesterol metabolism.

Additional studies are required to investigate the effect of LPS on the studied parameters in different brain regions and in sub cellular fractions.

8. SUMMARY AND CONCLUSION

In conclusion, the present study demonstrated that many of the symptoms that characterized some forms of depression in humans are presented in experimental animals following oxidative stress induced by LPS administration and then following it with acute stress. These symptoms included reduced social, exploratory and locomotor activity. Moreover, chronic treatment with hesperidin for 30 days attenuated or completely abolished these symptoms. These findings extend several other lines of evidence for the association between immune activation and depression, which were mentioned in the introduction. This study also provides a leading experimentation concerning the protective *in vivo* effects of hesperidin on various systems associated with oxidative stress in endotoxemic rat brain + acute stress and, that may provide excellent protection against several neurodegenerative diseases among other disorders associated with oxidative stress. Our data support the potential value of these agents for the therapy of septic shock.

Future studies should determine the molecular mechanisms that link LPS-induced HPA axis activation, physical stress and behavioral changes and the means by which hesperidin attenuate these responses. On the basis of published reports, and especially, on the ability of hesperidin to act as a potent anti-inflammatory agent, acting through diverse pathways, we can speculate that neuroprotective effects of hesperidin in LPS + acute stress-induced effects may be mediated by following distinct mechanisms: (1) inhibition of arachidonic acid metabolic pathways, PGE_2 and down regulation of COX and LOX (2) ability to enhance enzymatic and non-enzymatic antioxidant status (3) ability to act as scavengers of HESPERIDIN, (4) ability to attenuate the LPS-induced activation of HPA-axis (5) ability to normalize the levels of biogenic and acetylcholine neurotransmitter systems in brain (6) inhibit NO production (7) may counter the LPS-induced alterations of neuroendocrine, neuroimmune and neurochemical functions and stress hormonal levels.

The current report offers compelling, but perhaps not conclusive, arguments for an association between behavioral and brain anatomical changes induced by LPS and acute stress and its attenuation by hesperidin.

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